ANTIBIOTICS AND HUMAN PHAGOCYTIC CELLS

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DECLARATION

This thesis has been composed by me and the work, both clinical and in the laboratory is my own. The methods for studying leucocytes in the skin window preparations were developed in Leiden in collaboration with Professor Ralph van Furth and Miss Theda L. van Zwet during my tenure of a N.A.T.O. travelling fellowship. I have been assisted in countless ways by many colleagues whose names and the extent of their involvement are acknowledged on page 191 of this thesis.

26th March, 1976.

This thesis is dedicated to my family, especially my wife Patricia, my three children and my parents. They have all seen less of me than they should.

SPECIAL ACKNOWLEDGEMENT

Professor Ralph van Furth, University of Leiden introduced me to the fascinating activities of phagocytic cells. He encouraged me throughout an all too short sabbatical in Leiden and set aside countless hours for discussion of my results. Our conclusions sometimes differed but Professor van Furth had unlimited patience with an obstinate student. I alone must bear the responsibility for the results in this thesis.

ABSTRACT

1. The first studies of this thesis concerned human phagocytic cells *in vivo* as they responded to physical trauma in an experimental skin abrasion. This showed the sequence of cellular responses during acute inflammation and provided quantitative data about the phagocytic cell concentrations at each stage.

2. In an identical skin abrasion several antibiotics, administered systemically, were assayed. This provided information about the approximate concentration of each drug in acute inflammatory exudates.

3. A series of *in vitro* tests were performed to quantify the rate at which phagocytic cells, or the various antibiotics, could inactivate *Staphylococcus aureus* type 42b. The concentrations used were based on concentrations measured *in vivo* (paragraphs 1 and 2 above).

These studies showed that phagocytosis by human granulocytes is rapid ($T\frac{1}{2}$ = 5.6 minutes), but subsequent intracellular killing is much slower. In a cell-free system different antibiotics had variable rates of bacterial inactivation but, in general, these rates were of the same order as the rate of intracellular killing by normal/

normal human granulocytes. All these experiments utilised phagocytic cells and antibiotics separately.

4. The most important findings relate to the combined action of phagocytic cells and antibiotics (particularly ampicillin). The first clinical studies showed that during antibiotic therapy the rate of intracellular killing usually decreased. This effect could not be demonstrated by adding ampicillin in vitro to granulocytes during their phase of intracellular killing.

However, when ampicillin was administered orally to 4 healthy subjects there was a decrease of intracellular killing at 1 and 2 hours. The exact cause has not been established, but collaborative work indicates that this may be the result of inhibition by ampicillin of leucocyte myeloperoxidase and interference with other enzymes involved in the production or breakdown of intravacuolar hydrogen peroxide.

5. The importance of these findings is discussed in the light of a growing awareness that antibiotic therapy has considerable limitations and may have serious adverse effects.

ANTIBIOTICS AND HUMAN PHAGOCYTIC CELLS

GENERAL INDEX

ANONYMOUS GERMAN VERSE

"Sag Freund! Was ist den Theorie?"
"Wenn's klappen soll, und klappt doch nie."
"Und was ist Praxis?" - "Sei nicht dumm!
Wenn's klappt und keiner weiss warum"

(Anon.)

HISTORICAL REVIEW AND INTRODUCTION

CHAPTER I

HISTORICAL REVIEW AND INTRODUCTION

CHAPTER 1

"The longer you can look back,
the further you can look forward".
(Churchill, 1944).

Bacteria and Infection

In 1864 Louis Pasteur demonstrated that micro-organisms present in the atmosphere in low numbers could multiply in organic solutions such as wine or milk and cause fermentation (Vallery-Radot 1920). In the next four decades Robert Koch and his students showed that specific micro-organisms caused anthrax, diptheria, gonorrhoea, pneumococcal pneumonia and tuberculosis (Guthrie 1958). At the same time Armauer Hansen in Norway identified the bacillus causing leprosy (Guthrie 1958).

Immunity

In 1880 Chamberland and Pasteur noticed that attenuated cholera bacilli were not strongly pathogenic in chickens and that the birds which recovered then had immunity to virulent strains of cholera. Further studies with Emile Roux showed that attenuated anthrax strains /

strains could subsequently protect sheep from normally lethal doses of virulent anthrax bacilli (Reid 1974). Just as Jenner had observed that previous attacks of smallpox or infection with cowpox, either natural or artificial, could lead to long-lasting protection from smallpox (Jenner 1800), these more recent findings indicated that the micro-organisms which caused infection could sometimes be adequately controlled by the body and that subsequently the patient was immune to further challenge. Immunisation became an accepted treatment for infectious disease, particularly effective as a prophylaxis (Ransom 1896).

Theories of Immunity

Two apparently opposing views were put forward to explain immunity to infection. The first, the humoral theory, stated that immunity was dependent on the host producing antibodies which gave protection by causing neutralisation of toxins, and agglutination, precipitation or direct killing of the organisms (von Behring 1890, Pfeiffer 1894). The other theory, which emphasized the role of leucocytes, was based on the studies of Eli Metchnikoff, who had shown that in a wide range of animal species, the entry of micro-organisms would lead to a local accumulation of leucocytes which could phagocytose the invaders (Metchnikoff 1892). Almroth /

Almroth Wright, a Briton, then reconciled these two theories by demonstrating that serum factors, including antibodies, could greatly increase phagocytosis (Wright 1912).

The Therapy of Infection

Almroth Wright's dictum 'to stimulate the phagocytes,' was harshly parodied by George Bernard Shaw (The Doctors Dilemma, Shaw 1911) (but the medical literature appears to have largely ignored Shaw's irony e.g. Stossel 1974). We now understand that active immunisation can stimulate aspects of phagocytic cell function which are different from the secondary effects on these cells following the development of antibodies.

Serum therapy had a rational basis and was linked to the developing concepts of immunology. The principle was that an infected patient could have his immunity enhanced by the administration of an anti-serum active against the causative organism. Much of the work with antisera aimed at making these highly specific. However, quite separate methods of therapy soon took attention away from the immunological approach.

Joseph Lister (1867) had very rapidly applied

Pasteur's /

Pasteur's studies of the microbial aetiology of infection to the surgical situation. His method, based on the anti-bacterial action of solutions of carbolic acid, was dramatically effective, when used locally, in the prevention of wound infections. However, the toxic action of carbolic precluded its systemic use in man. In the 1890's the only possible systemic agents were antisera such as the diphtheria anti-toxin developed by von Behring (Reid 1974). Subsequently Ehrlich realised that many organic compounds could have a greater toxic effect on micro-organisms than on the host. In 1912 he showed that neosalvarsan, an arsenical, could be a reliable cure for syphilis.

The next milestone in the history of chemotherapy was the demonstration by Domagk in 1935 of the anti-streptococcal activity of prontosil, both in vitro and in vivo. However, the sulphonamide group of drugs such as prontosil were soon joined by penicillin, developed for clinical use by Florey, Chain and their colleagues (Florey et al 1941) based on Sir Alexander Fleming's in vitro observations (Fleming 1929). A common factor between the chemotherapeutic agents and the antibiotics, apart from their obvious antibacterial properties, is that their action can be explained without invoking involvement of the host's immune responses. Thus antibiotic /

antibiotic research and immunological research have followed different paths in the past three decades. The clinician tends to ignore immunological aspects when he prescribes antibiotic treatment for infection; his immunologist colleague pays scant attention to the effects of antibiotics upon his measurements of host responses.

Modern Antibiotic Therapy

The rapid increase in available antibiotics or chemotherapeutic agents since 1941 meant that virtually every bacterial species could now be attacked, *in vitro* at least. There are some mutant strains which are highly resistant to all therapeutic agents, but these may suffer biological disadvantages compared with the parent organisms (Mitchison 1954). However, Finland *et al* (1958) drew attention to the increasing incidence of drug-resistant gram negative infections which followed the liberal use of the early antibiotics.

In the 1960's *Pseudomonas aeruginosa* infection increased and caused life-threatening infection in widely different clinical situations. In a retrospective study of this infection, we found that the three commonest precipitating factors are:- serious underlying disease processes, previous infection with other /

other bacteria and treatment with antibiotics, particularly those with a broad spectrum. (Table 1:1 Raeburn, Geddes & Murdoch 1969). Even pseudomonas however, is susceptible *in vitro* to several antibiotics in concentrations which can be achieved in the serum, Reynolds *et al*, (1974) (table 1:2). In the 1970's infections with klebsiella species appear to have increased and to have added to the overall problem of antibiotic resistance. (Ayliffe, 1973).

The Development of New Antibiotics

To some extent the increase in antibiotic resistant infections has been paralleled by the introduction of new drugs (Garrod, 1970). Many of these were chemical modifications of older antibiotics which increased the activity against some species (e.g. substituted penicillins, Batchelor *et al* 1959). Other new agents were completely synthetic and were developed for their ability to block specific metabolic pathways in bacteria (e.g. trimethoprim, Hitchings 1969). Further antibiotics were discovered in a systematic search for anti-bacterial substances produced by individual micro-organisms (e.g. the cephalosporins, Abraham 1962). It is unlikely that the list of available antimicrobial agents will increase so rapidly again as in the past three decades.

Table 1:1

PRECIPITATING FACTORS IN PSEUDOMONAS INFECTIONS

(Based on a retrospective study of 117 patients*)

FACTOR	Number with factor	% with factor
Underlying disease	112	96
Associated bacterial infections	106	91
Antibiotics in preceding week	92	77

(* Raeburn, Geddes and Murdoch, 1969, Unpublished observations).

Table 1:2

MINIMUM INHIBITORY CONCENTRATIONS (M.I.C.)
OF ANTIBIOTICS AVAILABLE FOR PSEUDOMONAS INFECTIONS

Antibiotic	M.I.C. (mg/l)	Acceptable upper level in serum (mg/l)
Polymyxin B	1 - 16	10
Colistin	2 - 16	16
Carbenicillin	25 -100	500
Gentamicin	1 - 10	10
Ticarcillin	12.5 - 50	500
Tobramycin	0.5 - 2.5	10
Amikacin	1 - 20	16

Antibiotic Resistant Infections

Many microbiologists believe that bacterial resistance to the existing antibiotics will lead to an increase in certain bacterial infections (Gould, 1972, Philp and Spencer, 1974), but it is difficult to distinguish a selective increase of antibiotic resistant organisms from the other factors which influence the incidence of infection in a community, (table 1:3). However, it is a clinical impression that the increase of pseudomonas infection during the 1960's could not merely be attributed to the selective microbiological effects of antibiotics. At that time there was an increase in the number of severely ill patients; although their survival was often a medical triumph, such patients often had an increased susceptibility to infection. Antibiotics could have subtle effects on individual patients which alter either their host defences (Raeburn 1972) or affect the spread of micro-organisms between different individuals. There are many possible reasons for an increase in the number of infections in a community; the first sign of an adverse influence might be a relative increase of drug resistant infections.

Limitations of antibiotics

Some reasons for antibiotic failures in a particular patient /

Table 1:3.

SOME FACTORS AFFECTING THE DEVELOPMENT AND SPREAD OF DRUG RESISTANT INFECTIONS

1) The proportion of drug resistant organisms

2) The type of interaction between sensitive and resistant organisms

3) The effect of antibiotics on other bacteria

4) Host response factors: a) In individual patients
b) In the community

5) The opportunity for spread between different subjects

patient are summarised in table 1:4. Many studies show that resistance of the organism to the antibiotic used in treatment is not the only cause of such failures (Cluff et al 1968, McCabe & Jackson 1962, etc.) Inadequate absorption may occur or there may be poor distribution of the drug to the infected tissues. Impaired host responses will also predispose to failure of therapy, although if an active antibiotic can gain easy access to the organism, there should be measurable benefit during therapy, despite a degree of immune deficiency.

Table 1:4

CAUSES OF FAILURE DURING ANTIBIOTIC THERAPY

1) Bacterial resistance to the chosen antibiotic

2) Inadequate antibiotic access to infected sites:-

    a) Poor absorption

    b) Abnormal distribution

    c) Excessive excretion

3) Compromised host responses:-

    a) Locally - eg.

        i) Reduced drainage

        ii) Persistant foreign bodies

    b) Systemically - eg.

        i) Immunological disease

        ii) Metabolic abnormalities.

THE SEQUENCE OF HOST RESPONSES TO INFECTION

Acute Inflammation

Infection occurs when the skin or mucous surfaces of the host are breached and micro-organisms enter the sub-epithelial tissues. The infecting agents or their products stimulate an inflammatory response by causing the release from the tissue of endogenous mediators such as proteases, polypeptides or the pharmacologically active amines (Hurley, 1972). During the first hour of acute inflammation oedema develops as a result of increased vascular permeability and the resultant protein exudation (Spector & Willoughby 1972). Shortly afterwards polymorphonuclear leucocytes migrate into the area attracted by numerous chemotactic factors which include certain products of complement activation, especially C5a (Snyderman et al 1969) and C $\overline{567}$ (Ward, Cochrane & Müller-Eberhard 1965, Lachmann, Kay & Thompson 1970). There is a formidable list of other substances chemotactic for neutrophils, the majority of these being proteins or polypeptides (Wilkinson 1974). Eight hours after the onset of tissue trauma, up to 50% of the cells of the exudate are characteristic mononuclear phagocytes and thereafter these cells predominate. (see appendix p 183).

Phagocytosis /

Phagocytosis and Intracellular Killing

The acute inflammatory reaction rapidly leads to an accumulation in infected sites of phagocytic cells, initially neutrophils and then monocytes, which can engulf the causative organisms and later inactivate them. In addition, inflammation provides certain antibodies and complement components which, by opsoninising bacteria, render them more easily ingested. It is very important to make a clear distinction between the phagocytic process and the subsequent bactericidal action. Phagocytosis is usually rapid and depends on energy derived from glycolysis (Karnovsky 1962). Intracellular killing of ingested bacteria may be a slower process and derives the necessary energy from aerobic metabolic pathways; until killing is complete the host remains at risk of recrudescence of infection. From the studies of Holmes et al., (1966) and Solberg (1972a) it can be concluded that phagocytosed organisms are protected from the effects of antibiotics which tend to remain extracellular. Intracellular organisms will have already been confronted by antibody, complement and other serum factors. Nevertheless some serum is also necessary during the phase of intracellular killing.

Varieties /

Varieties of Phagocytic Cell

A degree of phagocytosis can be achieved by other cells besides granulocytes and monocytes. (Rabinovitch 1968). The distinction of granulocytes and monocytes is the rapidity of phagocytosis and its extent and also the ability to ingest, preferentially, particles which have been coated with immunoglobulin such as IgG or an IgM - complement complex. Recent studies have demonstrated that IgG and C3 receptor sites are present on the cell surfaces of both mononuclear phagocytes and neutrophils but there are differences in the behaviour of the receptors (Baehner, 1975).

Phagocytosis can take place in anaerobic conditions but when the particles are being phagocytosed, aerobic metabolic pathways are stimulated in preparation for the metabolic activities which accomplish intracellular killing. Confusion occurs unless it is understood that these aerobic pathways are 'triggered off' during phagocytosis as an effect and are not the cause. If, during phagocytosis, aerobic activity cannot take place then subsequent intracellular killing is greatly reduced, (Baehner, 1975, Mandell, 1974). There are several syndromes of decreased intracellular killing in the presence of normal phagocytosis, (Raeburn 1975), which will be detailed later on.

Acquired /

Acquired Cellular Immunity in Host Resistance

Two easily distinguishable phagocytic cells participate in the acute inflammatory response and it would be strange if their functions were identical. The presence of mononuclear phagocytes is closely linked to the development and manifestations of specific cellular immunity (Mackaness 1970). After recovery from an infection with any of a variety of intracellular organisms the macrophages are found to be morphologically altered, to contain more lysosomal granules and to show more pinocytosis (van Furth 1972). These changed macrophages can also show increased phagocytic activity (Lurie 1964) along with enhanced microbicidal action (Blanden 1968) and have been appropriately described as "activated macrophages" (Mackaness 1970). Some time later the number of activated macrophages decreases until further infection with the same organisms occurs whereupon it increases much more rapidly than after the first challenge due inter alia, to the action of lymphokines produced by sensitised lymphocytes (Dumonde et al 1975). The specificity lies in the stimulus which leads to activation. After activation the cells have increased non-specific activity against many other intracellular organisms.

Antibody in Host Resistance /

Antibody in Host Resistance

The division of lymphocytes into two main groups of 'B' and 'T' cells, is a gross over-simplification but it is convenient. B cells produce specific anti-bacterial antibody which plays a major role in the continuing host defences against infection. T cells release lymphokines such as blastogenic factors which can stimulate B cells thus increasing antibody formation (David 1971), or can activate macrophages (Dumonde et al 1975). Present evidence indicates that circulating lymphocytes can permeate most tissues and would thus reach a site of bacterial infection. There, or in local lymph nodes, specifically sensitised B lymphocytes can differentiate into plasma cells and produce antibody.

In vitro techniques have shown that normal human peripheral blood lymphocytes can synthesize IgG and to a lesser extent IgA and IgM (van Furth et al., 1966). Similar techniques have been used to study immunoglobulin synthesis in other human tissues such as skin (Lai, 1973), mucous membrane or parts of the gastro-intestinal tract (McClelland et al., 1974).

General Aspects of Infection and Immunity

The activities of the cells of the immune system provide a co-ordinated sequence of defences against infection. Phagocytic cells are active at an early stage as are a variety of non-specific serum factors such /

such as proteases, lysozyme or transferrin. The phagocytic cell response becomes predominantly monocytic after a few hours and these cells subsequently cooperate with a variety of lymphoid cells to initiate specific immune responses. The cellular activities at each phase are summarised in tablel:5.

Table 1:5

THE SEQUENCE OF CELLULAR ACTIVITIES

DURING INFECTION

	CELL INVOLVED	ACTION	TIME AFTER INFECTION (HRS)
A.	Infecting bacteria	Toxin release	0 →
B.	Tissue cell targets	Release of intracellular enzymes etc.	0 →
C.	Granulocytes	Phagocytosis & killing	1 - 24
D.	Monocytes ↓ Macrophages	As in C + Antigen processing etc.	6 - 72
E.	Fibroblasts	Wound repair	48 - 500
F.	Lymphocytes	Specific immune responses	48 →

SUMMARY OF CHAPTER 1

1. The first approaches to the treatment of microbial infection were based on the administration of immune sera, active immunisation or other means of enhancing immunity. The host response to infection is initially dependent on phagocytic cells complemented by a variety of serum factors. Later a coordinated and specific immune response provides complementary means of controlling micro-organisms.

2. Antibiotic therapy appears to control bacterial infections without the need for stimulation of normal immune responses. Conventional antibiotic regimes do not necessarily provide the most useful reinforcement of the natural host defences.

3. The following chapters summarise a series of studies to elucidate how antibiotic therapy can best be made to complement these activities of the immune system.

PHAGOCYTIC CELLS IN THE INITIAL RESPONSES TO INFECTION

CHAPTER II

PHAGOCYTIC CELLS IN THE INITIAL RESPONSES TO INFECTION

CHAPTER II

"As bees bizz out wi' angry fyke,
when plundering herds assail their byke,"
(Burns, c. 1785).

Introduction:

The Inflammatory Process

Many centuries after Celsus defined the cardinal signs of inflammation as rubor, tumor, calor & dolor, (c.A.D. 30) John Hunter (1794) suggested that inflammation might well be 'salutory' and an important part of the body's defence against injury. Lister (1858) and Cohnheim (1882) described the changes occurring during inflammation and then De Haan (1920) showed that the first leucocytes in acute inflammatory exudates were granulocytes which, after the first few hours, were partially replaced by mononuclear cells. Rebuck & Crowley (1955) described the skin window technique for studying inflammation in vivo in man but erroneously suggested that the mononuclear cells present after a few hours were derived from lymphocytes. The skin window technique has been used /

used here to examine, in this chapter, the sequence of the cellular responses and in chapter three the localisation of administered antibiotics, following acute inflammation induced by minimal physical trauma.

METHODS

Reasons for using the Skin Window technique

The skin window technique provides an accessible model of the acute inflammatory response and can be used without danger or undue discomfort to the subject. The cellular changes seem very similar to those which occur during acute inflammation in the lungs (Boyd, 1965) or the kidney (Robbins, 1967), For this thesis, skin window studies provide a practical means of examining the early relationship between phagocytic cell activities and antibiotic therapy. In addition, a variety of serum factors can be examined. Very recently MacClelland & van Furth (1975) have measured immunoglobulins, albumin, lysozyme and complement in the resultant exudates.

The Skin Window technique

Initial experiments showed that the middle of the non-dominant forearm was a suitable site for use in the test and that during each experiment the subjects could move/

move around. The chosen area was shaved and then cleansed with 70% alcohol which was allowed to evaporate completely. Initially Abrasions were made with a sterile scalpel, the blade of which was held vertical and gently scraped from side to side over the tensed, horizontal skin. An alternative method in which the tensed area was touched lightly with a high speed rotating sterile buff (fig. 2:1) proved more rapid, less painful and gave a more consistent experimental lesion. This technique was adopted for all the subsequent studies. The area abraded in each case ranged from 5 x 5mm square to 5 x 10mm and could most easily be measured after 24 hours when a scab had formed. At the macroscopic level these lesions showed a sticky exudate for three or four hours with mild erythema extending slightly beyond the lesion. The scab (fig 2:2) became detached in the subsequent 48 hours to leave a faint red scar which had faded within a few weeks.

Collection of the exudate

Three different methods of collection were used, each suitable for a particular purpose.

1. Sterile glass coverslips, 10mm in diameter, were applied directly to the lesion and covered with a 12mm filter paper disc, fixed in place with micropore tape. This method was best for collecting phagocytic cells /

Figure 2:1 High speed drill and sterile buff being used to make a skin abrasion.

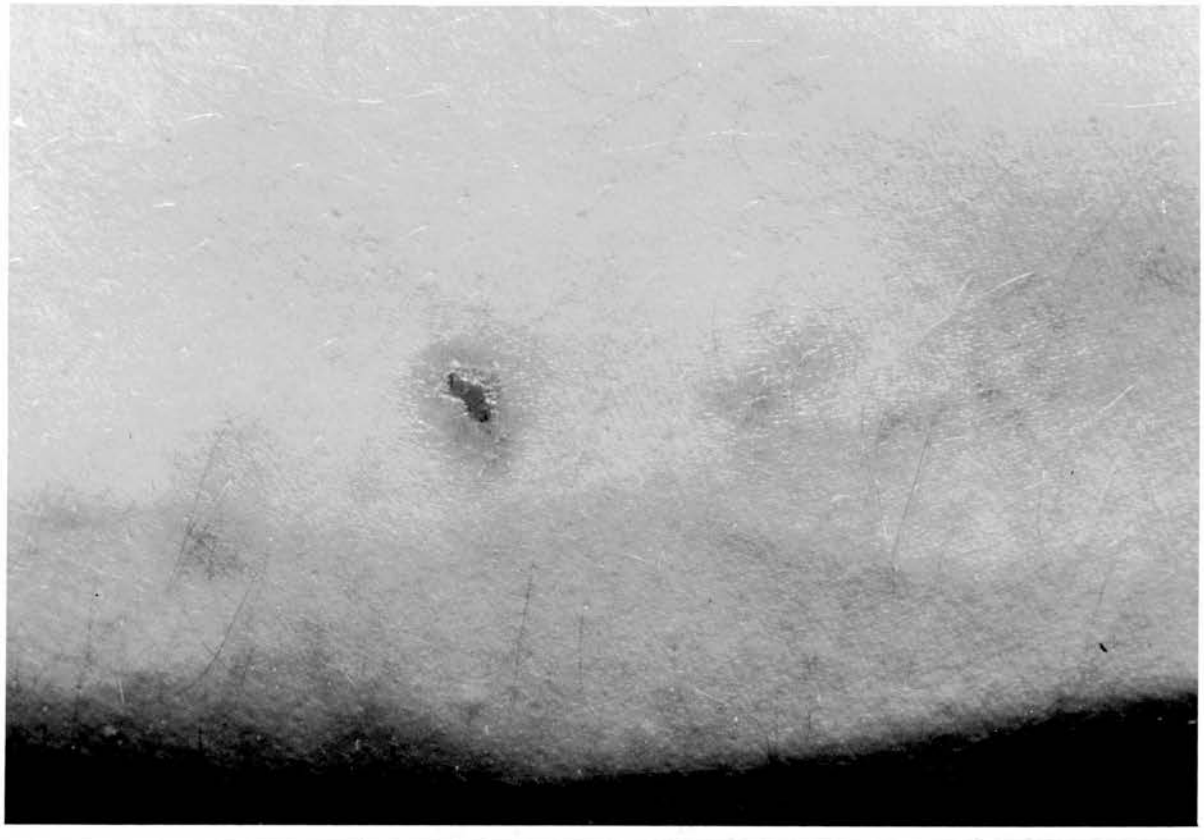
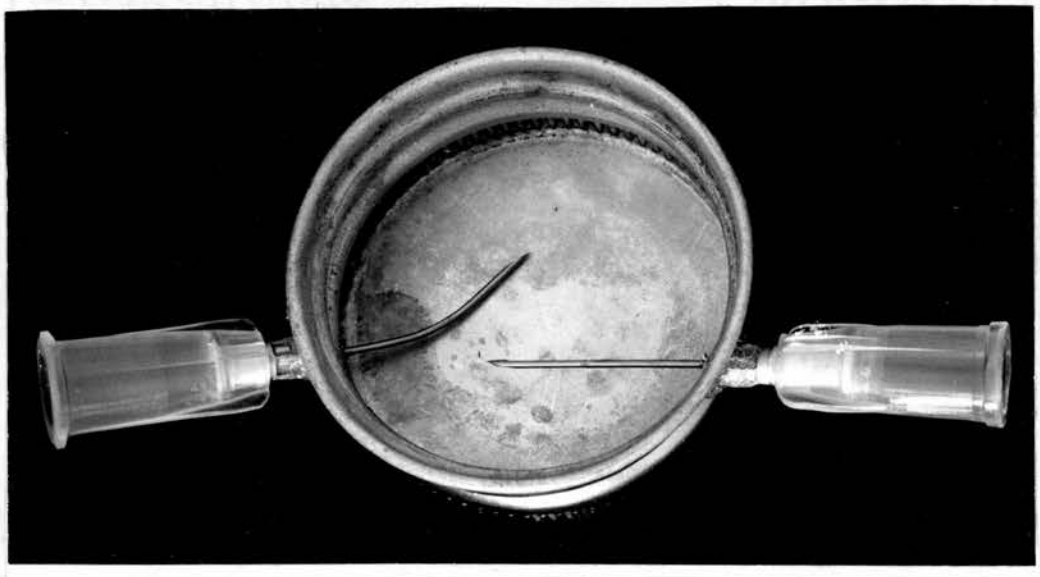


Figure 2:2 Skin abrasion after 24 hours showing small scab.

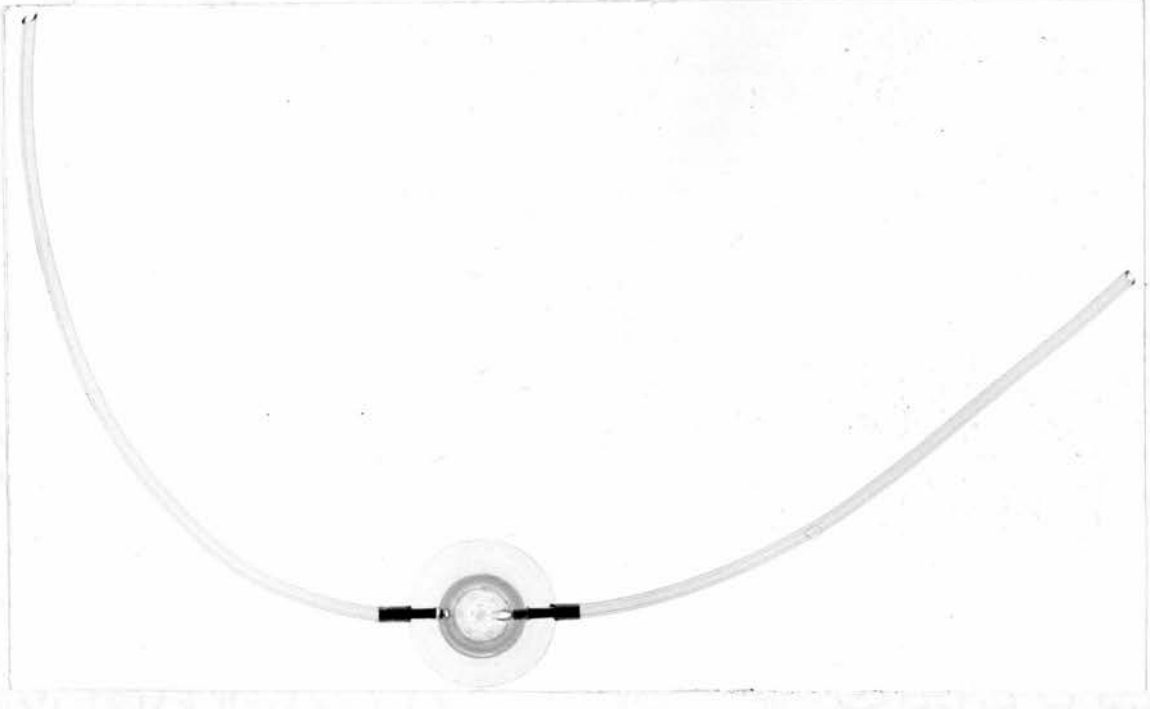
cells which adhered to the glass and could then be subjected to morphological, histochemical or functional tests. Cells could be collected in this way for up to 48 hours and while still attached to the glass, could be cultured *in vitro*.

2. The lesion was covered with a variety of chambers (see figs 2:3, a & b), which were fixed in position using both collodion around the periphery and pressure applied with a wide strip of micropore. The chamber was filled with medium 199 which was usually changed every 6 hours. This method was used for collecting cells so that the total and differential leucocyte count could be made.

3. The exudate was taken up by sterile filter paper discs of either 6 mm or 12 mm diameter placed directly over the abrasion. These were weighed before use and immediately afterwards. During contact with the lesion, the discs were covered with glass microscope slides (fig. 2:4) which had smoothly ground edges to avoid chafing. The slides were kept firmly in position by micropore tape. It was found that the discs did not move appreciably during the first 3 or 4 hours of inflammation because of the sticky exudate. Later it was difficult to keep the discs in place. This method produced/



a.



b.

Figure 2:3 Two chambers used in method 2. Volumes were 2.1 ml. for (chamber a) and 0.5 ml. (chamber b).

See appendix 6 page 211

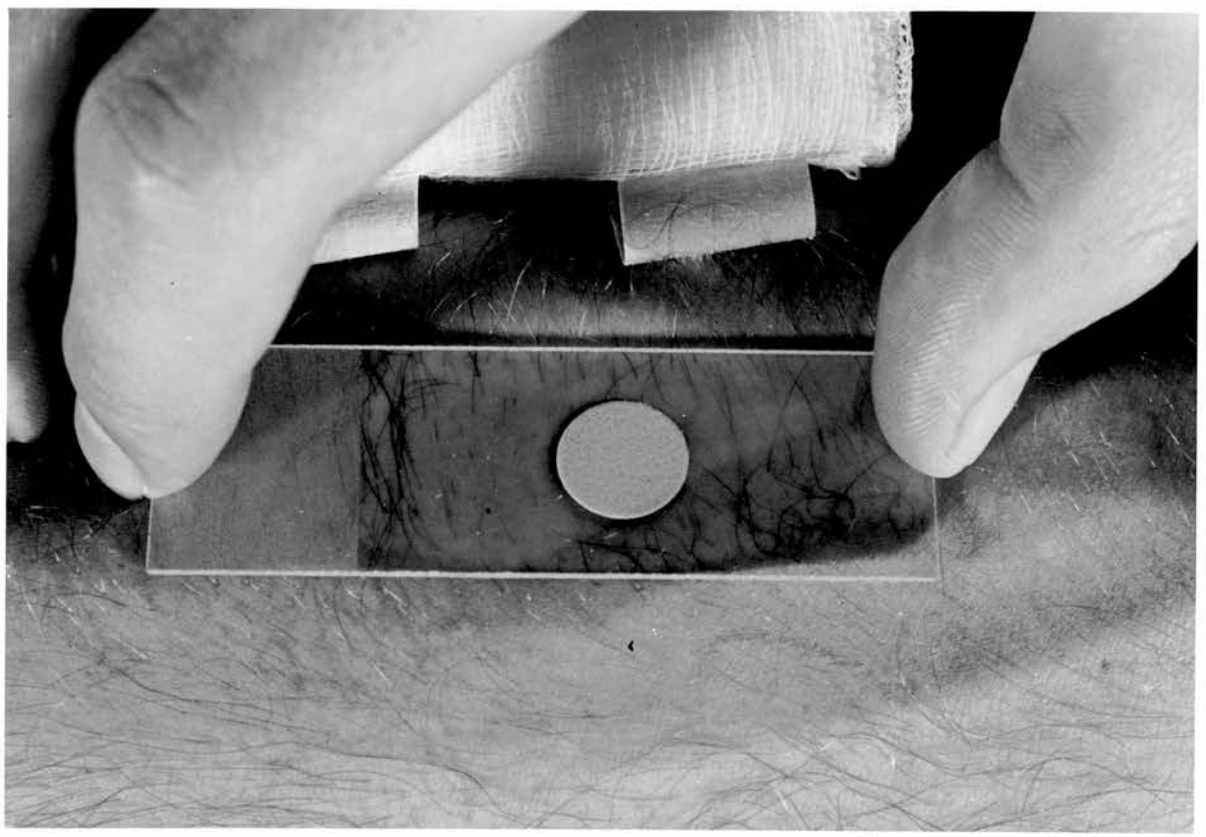


Figure 2:4 Method for collecting exudate on sterile filter paper discs, covered by a prepared microscope slide.

produced samples of exudate varying from 2 to 20mg in weight which were used for antibiotic assay (Chapter 3).

Time of sampling

In these experiments, many factors influenced the duration of each sampling period. For the study of phagocytic cells, the limiting factor was the degree of confluence on the coverslip. If a particular coverslip was in position for more than 3 hours the cells were so closely packed that differential counts could not be performed. For the studies of antibiotics, at least 1 hours collection was required to have sufficient exudate for assay.

Other Methods used in Leucocyte Studies

i)* Cell counts were performed using the Neubauer counting chamber and white cell diluent consisting of 1.5% glacial acetic acid plus 0.01% gentian violet (v/v), in distilled water.

ii) Exudate leucocytes adherent to the coverslips (Method 1) were studied as follows:-

a) The /

* These studies were performed with Miss Wilma Jack, a third year medical student.

a) The coverslips were changed regularly, dried rapidly by fanning, and fixed in absolute methanol for 15 minutes. They were stained for 10 - 15 minutes (depending on the density of cells) in Giemsa, diluted 1 in 10 in distilled water and mounted face uppermost on standard glass microscope slides using Depex. On these preparations polymorphonuclear leucocytes were easily identified, being morphologically identical to the same cells in the peripheral blood (fig 2:5).

b) Since the mononuclear cells of the exudate were often morphologically different from any leucocyte of the peripheral blood a series of cytochemical and functional tests were performed to identify their origin (Appendix 1). Since these studies were dependent on maintaining the cell viability the coverslips were processed as follows:-

Before removal from the abrasion approximately 0.25 ml. of sterile medium 199 was injected under the edge of the coverslip.
This made the coverslip float up from the abrasion/

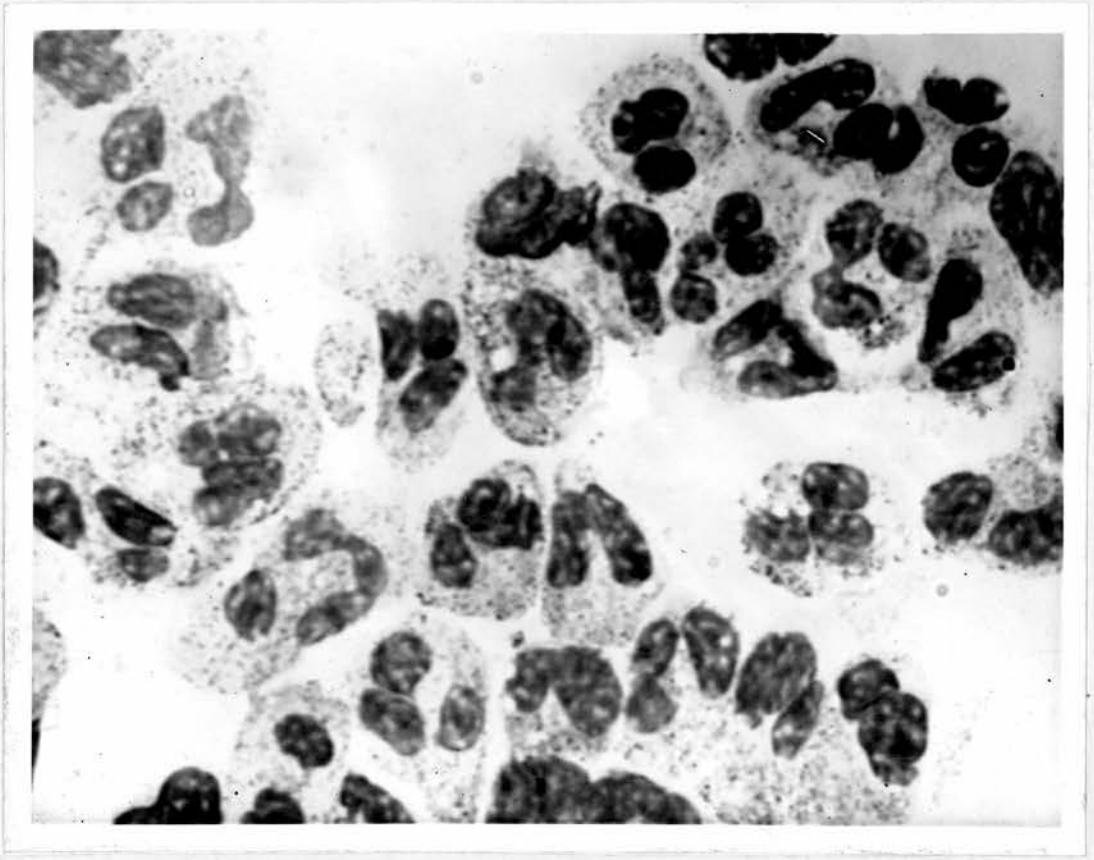


Figure 2:5 Polymorphonuclear leucocytes in a skin window preparation at 4 hours (x 1000). (Giemsa stain)

abrasion so that it could be gently lifted from the site with the cells and the medium on the under surface. The coverslip was then placed (cells uppermost) in 50mm petri dishes containing medium and serum suitable for the subsequent tests.

RESULTS

Leucocyte Counts in Normal Exudates

The chamber method (method 2) shows that in hospital controls an abrasion of 25mm^2 can accumulate at least 1 x 10^6 leucocytes in a 24 hour period. Table 2:1 gives some clinical data and the individual results. The considerable variation between tests reflects in part the difficulty in standardising the depth of the skin lesion. The degree of reproducibility in one individual subject was not studied. Table 2:2 shows that during the first six hours the mean hourly number of cells mobilised was 0.04 x 10^6. In suceeding sampling periods the mean number of cells mobilised per hour increased.

During the first 2 hours of inflammation there were very few leucocytes of any type and the chamber method was /

Table 2:1

TOTAL LEUCOCYTES IN SKIN WINDOW CHAMBERS

IN HOSPITAL CONTROLS*

Subject	Age	Disease *	Leuco-cytes/ 24 hours ($\times 10^6$)	Mean rate/hr ($\times 10^6$)
W.A.	38	Undiagnosed chest pain	3.57	0.15
G.B.	59	Myocarditis	13.46	0.56
H.B.	25	Coal gas poisoning	21.10	0.87
A.C.	44	Barbiturate overdose	13.13	0.55
J.C.	31	Paracetamol overdose	14.17	0.59
J.I.	54	Hypertension	48.40	2.02
D.J.	20	Aspirin overdose	13.26	0.55
M.J.	36	Barbiturate overdose	1.79	0.07
D,M.	35	Aspirin overdose	1.25	0.05
W.M.	49	Mercuric chloride poisoning	2.90	0.12
C.S .	36	Amitryptiline overdose	2.70	0.11
		MEANS	12.34	0.51
		STANDARD ERRORS	4.13	0.17

* All tests performed when patient was ambulant and about to be discharged.

Table 2:2

TOTAL LEUCOCYTES IN SKIN WINDOW CHAMBERS (MEAN MOBILISATION RATE (PER HOUR) DURING DIFFERENT COLLECTION PERIODS) [x]

Time of collection	No. of experiments	Mean leucocytes/ hour
0 - 6 hours	3	0.04×10^6
0 - 12 hours	5	0.15×10^6
0 - 18 hours	5	0.19×10^6
0 - 24 hours	11	0.51×10^6
		(S.E.M. 0.17)

[x] In some subjects the chamber fluid could not be changed 6-hourly and therefore the later cellular influx was 'diluted'. For the other subjects cell counts at each period were summated to give overall figures for 0 - 12, 0 - 18 or 0 - 24 hours.

was therefore unsatisfactory for counting. However, in separate experiments, it was possible to count the total "glass adherent" cells during the first 2 hours of inflammation from coverslip preparations, (Method 1).

These numbers and differential counts are shown separately in Table 2:3. After 2 hours the cells were too numerous and too confluent on coverslips for accurate counting.

The Nature of Exudate Cells in Normal Subjects

Polymorphonuclear leucocytes were, by far, the most common cells identified in the early stages of inflammation. At two hours they comprised over 95% of the cell population, falling to 80% at 4 hours and 60% at 6 hours. As Table 2:4 shows, the skin macrophage was the cell which increased in the preparations as the polymorphonuclear proportion fell. Figure 2:6 shows the proportions of these two cell types in one individual throughout the first 24 hours. Figures 2:5 & 2:7 shows representative high power fields at different time periods. Lymphocytes were very rarely present in the coverslip preparations. Since this may have been an artefact due to the low glass adhesiveness of lymphocytes, a few preparations of the cells mobilised into/

Table 2:3

TOTAL LEUCOCYTES ON COVERSLIP PREPARATIONS FROM SKIN WINDOW EXUDATES DURING THE FIRST 2 HOURS*

Subject	Age	Total cells	Differential (%) Granulo-cytes	lympho-cytes	mono-cytes
B. McC.	28	417	100	0	0
S.R.	31	85	95	2	2
Dv. W.	29	106	94	0	6
Th. v.	35	49	94	4	2
	MEANS	164	98	0.6	1.4

* 4 normal subjects

Table 2:4

LEUCOCYTE TYPES PRESENT IN SKIN WINDOWS AT VARYING TIMES AFTER AN INFLAMMATORY STIMULUS*

Time after abrasion (Hrs)	Cell Type (%)			S.E.M.
	Granulocytes	Lymphocytes	Skin window macrophages	
0-2	95.4	0	4.6	2.1
2-4	79.4	0	20.6	6.0
4-6	60.4	1.4	38.2	10.3
6-8	53.3	0	46.7	9.2
8-10	42.7	0	57.3	11.0
10-24	24.9	1.6	73.5	14.1
Over 24	40.0	0.5	59.5	12.6

* Means of studies on 6 normal subjects.

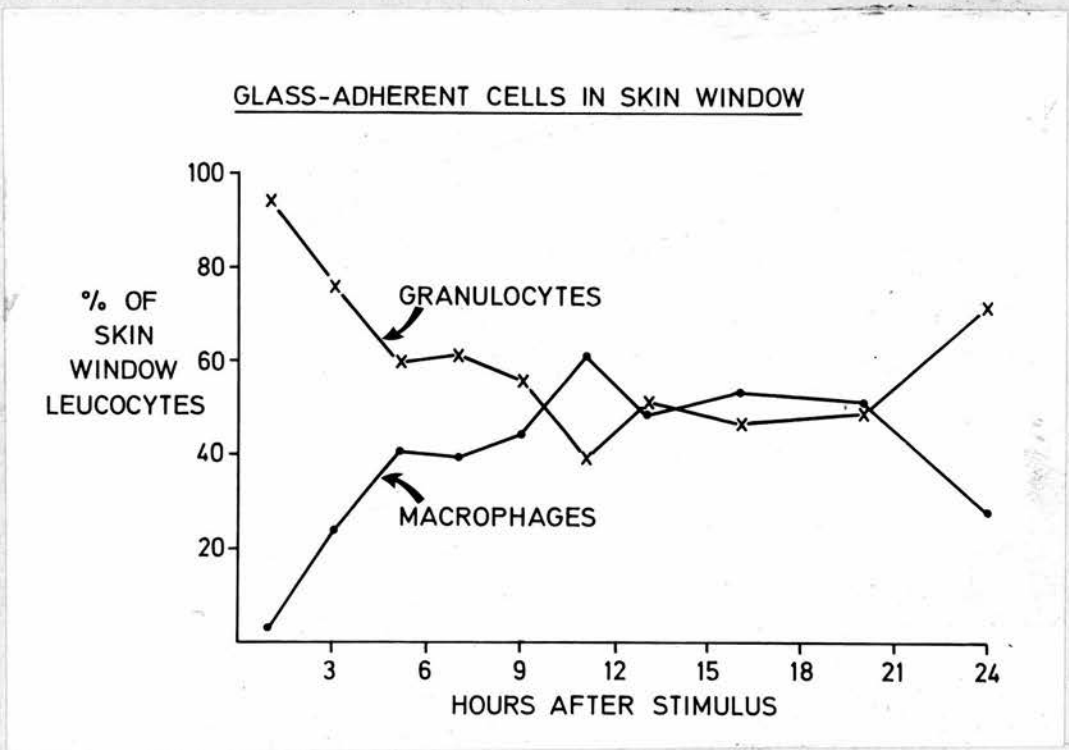


Figure 2:6 Skin window cell types in one normal subject during the 24 hour period following an abrasion.

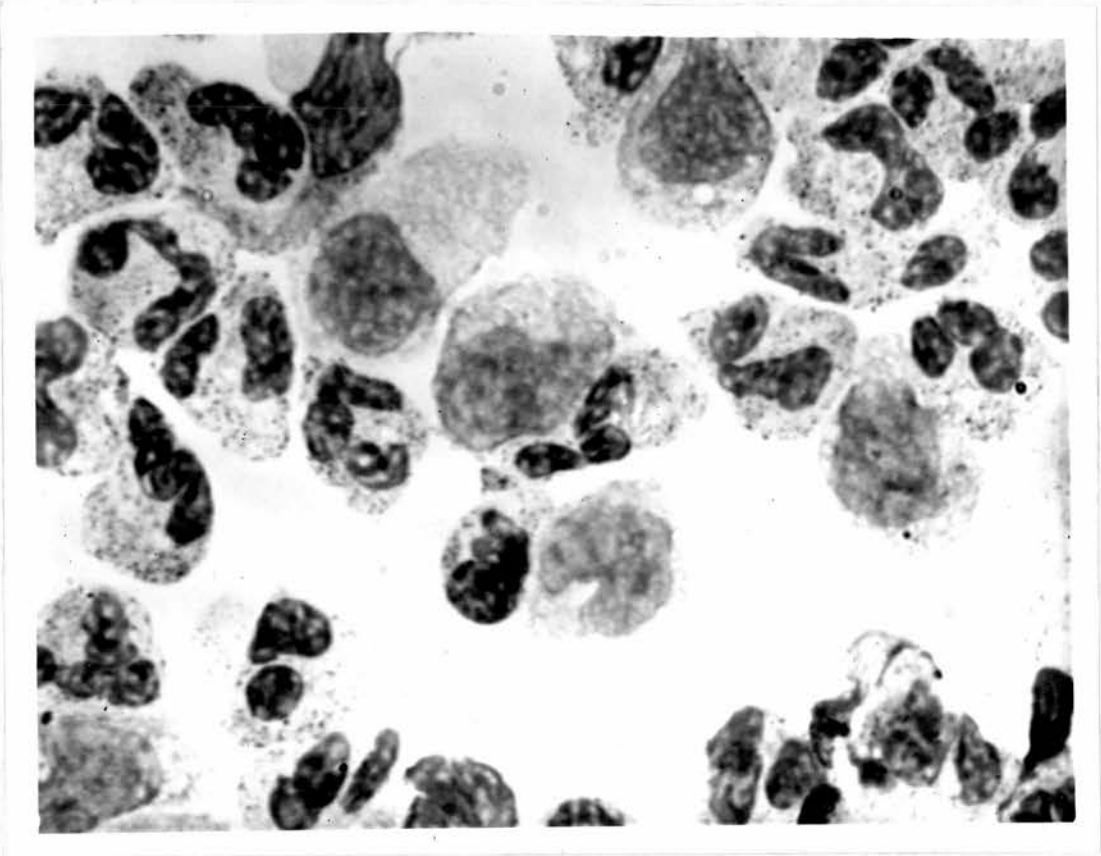


Figure 2:7 High power view (x 1000) of skin window cells at 8 hours showing the polymorphonuclears and the skin window macrophages. (Giemsa)

into skin chambers (method 2) were also examined. These showed very few lymphoid cells, identified by morphology after Giemsa staining.

Leucocytes in skin windows of various patients

1. Acute leukaemia

Studies were performed on two patients with acute myeloblastic leukaemia and two with acute lymphoblastic leukaemia using method 1. In all four cases there was a gross diminution in the number of cells mobilised in 24 hours (table 2:5).

2. Chronic lymphatic leukaemia

Five patients had skin window studies, primarily to examine the kinetics of antibiotics. However, in the period from 3 to 24 hours coverslips were applied to examine the cellularity of the response. Accurate counting was not possible but these patients in contrast to normals had at least 50% lymphocytes in the exudate. (Fig. 2:8).

3. Chronic benign neutropenia

One female infant had a skin window performed when the peripheral neutrophil count /

Table 2:5

TOTAL GLASS-ADHERENT CELLS IN PATIENTS WITH ACUTE LEUKAEMIA

Patient	Diagnosis	Age	Total cells[x] in 24 hours
J.D.	ALL	58	8×10^3
M.F.	ALL	73	5×10^4
J.T.	AML	48	5×10^1
J.B.	AML	38	18×10^2

x All the cells present on a glass coverslip held in contact for 24 hours after the skin abrasion were counted. The morphology (Giemsa stain) was not normal and differential counts were not attempted. However, a higher proportion of granulocytes was present than in the peripheral blood.

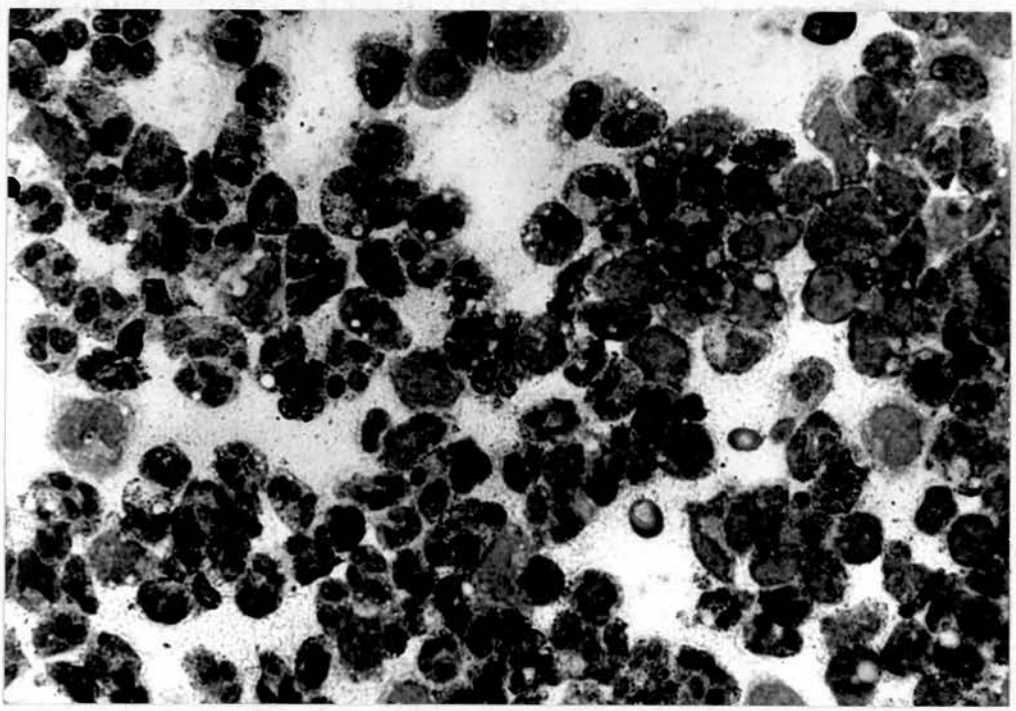


Figure 2:8 Skin window preparation from a patient with chronic lymphatic leukaemia (3 - 24 hour sample). Note the proportion of lymphocytes present.
(Giemsa stain x 550)

count was less than 300/mm^3. No cellular response occurred; in particular skin window macrophages did not seem to replace the neutrophils in the reaction. The peripheral blood count of monocytes was about 600/mm^3 at this period.

DISCUSSION

Total Leucocytes in Skin Abrasions

In our hospital control subjects the total leucocytes counted in chamber fluid over an abrasion ranged from 1.3 x 10^6 to 48.4 x 10^6 in 2 4 hours with a mean of 12 x 10^6. This figure can be compared to the findings of Holland et al (1971) in 46 healthy subjects. With an abrasion of roughly 100mm^2 they found a mean mobilisation rate of 76.0 x 10^6 in 24 hours and a range from 45 x 10^6 to 130 x 10^6. Perillie & Finch (1964) had found that normal subjects could mobilise 49.2 x 10^6 leucocytes in 24 hours to an abrasion of 400mm^2. After correction for differences in abrasion size and allowing for their use of serum in the chambers with its greater chemotactic activity, these findings and our own are broadly similar.

Leucocyte /

Leucocyte Types in Skin Abrasions

Our studies with coverslips on the skin abrasion showed a gross preponderance of granulocytes in the first 4 hours, a gradual increase of skin window macrophages thereafter, and at 8 hours macrophages predominated. This finding contrasts sharply with the skin chamber results of Holland et al. (1971); their studies showed that the average percentage of macrophages during 24 hours was between 0 and 10%. Perillie & Finch (1964) reported that polymorphonuclear leucocytes "predominated at all stages" but did not give figures. Kowal (1969) reported that using a coverslip technique the percentage of mononuclear cells (not clearly specified) rose to about 50% at 12 hours. Thus there appears to be a difference between the type of leucocytes which adhere to glass and those which enter a skin chamber. Since cytochemical studies were not performed in our early skin chamber studies, this aspect remains unclear. However, both in skin chambers (Holland et al. 1971, Perillie & Finch 1964) and in glass-adherent methods (Dale & Wolff 1971, Braunsteiner & Schmalzl 1970, Wulff 1967,) there is a consensus that lymphocytes are very infrequently seen in skin window inflammatory exudates. The /

The phagocytic cells predominate during the first 24 hours at least. This initial high concentration of phagocytic cells is logically explained as the provision of an immediate defense against invading bacteria.

Concentration of Phagocytic Cells in Skin Window Exudates

The relative concentration of bacteria and phagocytes is of importance in determining the rate of ingestion (van Furth & van Zwet 1973). Skin chamber techniques alone do not allow any measurement of phagocyte concentration since the exudate volume, grossly diluted with chamber fluid, cannot be measured. However, by calculations from the mean of hourly exudate weights found by method 3 (page29) and the hourly mobilisation rates at different stages (table 2:2), some approximation to the actual *in vivo* concentrations can be deduced (table 2:6). Thus, in studies with phagocytic cells *in vitro* it would be realistic to use concentrations in the range 3.8×10^6/ml to 5.0×10^7/ml. These estimations can be a basis for developing *in vitro* models of bacteria/phagocyte interactions which closely resemble the *in vivo* situation (Chapter 4).

Leucocyte /

Table 2:6

CALCULATED PHAGOCYTIC CELL CONCENTRATIONS IN SKIN WINDOW PREPARATIONS[x]

Time	Hourly cell count	Calculated cell concentration/ml.*
0 - 6 hours	4×10^4	3.8×10^6
0 - 12 hours	15×10^4	1.4×10^7
0 - 18 hours	19×10^4	1.8×10^7
0 - 24 hours	51×10^4	5.0×10^7

x Based on figures from table 2:2 on an assumed hourly exudate volume of 10.5 micro litres. (See text page 48)

Leucocyte Mobilisation in Blood Disorders

No attempt has been made to study large numbers of patients with leukaemia or other haematological disorders. However, the data in table 2:5 is in keeping with the findings of Holland et al. (1971) and of Perillie & Finch (1964) that patients with acute leukaemia mobilise fewer leucocytes to skin abrasion sites than healthy controls.

The qualitative findings in patients with chronic lymphatic leukaemia correspond to those of Kowal (1969) who found that mononuclear cells in these exudates were abnormally increased. Our patient with chronic familial neutropenia was probably unique and is noteworthy from many points of view including the presence of leucoagglutinins, (see Kay et al 1976). However, the gross diminution of the skin window response and the relative lack of skin window macrophages contrasts with the findings in several types of neutropenia reported by Dale & Wolff (1971).

Further studies of leucocyte mobilisation in different disease states are required to determine for instance whether reduced mobilisation indicates a need for more active therapy of any infection than would otherwise be used.

SUMMARY OF CHAPTER 2

U p to 48 x 10^6 phagocytic cells can be mobilised into an experimental skin lesion of 25mm^2 area, during the first 24 hours of inflammation as measured in skin chambers. The coverslip method shows that these cells are initially almost entirely polymorphonuclear leucocytes but that after 6 hours the mononuclear phagocytes increase to become the predominant cell.

Three patients with acute leukaemia had gross diminution of the inflammatory responses to an extent which would be likely to predispose to infection. Although five patients with chronic lymphatic leukaemia had a reasonable cellular response (not accurately estimated), the leukaemic lymphoid cells could be seen in the exudate as well as normal neutrophils and skin window macrophages. A patient with congenital neutropenia had gross reduction of both types of phagocytic cell in the exudate.

These findings provide a basis for examining the antibacterial action of phagocytic cells *in vitro*, in concentrations similar to those achieved during *in vivo* inflammation.

ANTIBIOTICS IN THE INFLAMMATORY RESPONSE

CHAPTER 3

INTRODUCTION

ANTIBIOTICS IN THE INFLAMMATORY RESPONSE

CHAPTER 3

"---- it occurred to me that decomposition in the injured part might be avoided by applying ----- some material capable of destroying the life of the floating particles"

(Joseph Lister, 1867).

INTRODUCTION

The efficiency of phagocytic cells is initially determined by their ability to move rapidly to areas of infection. Likewise antibiotics cannot act beneficially unless they achieve adequate concentrations at infected sites within a short time. A method for examining antibiotic concentrations in areas of skin inflammation has been developed in this work. This chapter gives the results of several studies with antibiotics in skin window exudates from healthy or leukaemic patients.

THE SKIN WINDOW METHOD FOR ANTIBIOTIC STUDIES

The preceding chapter described the technique for making skin abrasions and for sampling both cells and exudate /

exudate fluid. In the following studies antibiotics were administered* prior to making the skin lesions and hourly collections of exudate were made for three hours. Blood for assay was taken at the mid-point of each hourly exudate collection period so that some comparison between serum and exudate concentrations could be made. The method for exudate collection has already been described (page 29 method 3, and figure 2:4)

METHODS FOR ANTIBIOTIC ASSAY

Some antibiotics or chemotherapeutic agents can be estimated using chemical techniques but these methods are not sufficiently sensitive to assay concentrations below 1 mg/l or small samples. Moreover, each agent requires different extractions prior to the chemical assay and this makes comparisons between different drugs difficult.

The microbiological assay method has been well tested as a means of antibiotic measurement and has a flexibility suited to the purpose of this study. The /

* Oral drugs were given 30 minutes before the skin trauma, intramuscular drugs 15 minutes before.

The principle is that a test organism, susceptible to the drug under study, is incubated in media containing either standard drug concentrations or a known dilution of the test fluid. From the relationship between the organism's growth in the unknown or the standard mixture, the test concentration can be estimated. There are two main methods for microbiological assay (Garrod & O'Grady, 1968). In the simplest the test is performed in fluid media containing different dilutions of either the standard or test solutions. The end point would be the lowest dilution of either which inhibits growth. Thereafter the approximate concentration of the sample can be calculated.

The other method uses solid media which have been inoculated throughout with suitable bacteria. Test or standard solutions are applied to these preparations and the end point is measured as the diameter of the resultant inhibitory zones following several hours culture.

There are several advantages of the solid medium method, particularly that it enables levels which fall between the standard dilutions to be estimated. In addition, since contaminating bacteria within the test/

test sample can be distinguished from the assay organisms, assays are possible in infected fluids such as sputum. Above all, repeated comparisons of assay techniques conducted on a national scale have shown consistently that the plate diffusion method is more accurate in practice than tube dilution assays (Reeves & Bywater, 1975).

After several preliminary tests the following method was found to be satisfactory for the assay of all the relevant antibiotics.

THE PLATE DIFFUSION ASSAY METHOD

Five hundred mls. of agar medium (A/B medium no. 2, Oxoid), prepared at a pH suitable for the chosen antibiotic, were sterilised for 15 minutes at 120°C and a pressure of 15 pounds per square inch. The resultant solution was allowed to cool to 48°C and kept in the molten state at this temperature. The test bacteria[x] were added and the flasks were carefully agitated. Ten ml. aliquots were pipetted into 9 cm. diameter Petri dishes and allowed to cool and solidify on a levelling table. The antibiotic solutions were either pipetted into round wells (punched in the seeded medium with a 4 mm/

x For details of the sources of and methods to maintain the assay bacteria please see appendix 6, pages 211 & 212.

4 mm diameter suction cork-borer) or applied to filter paper assay discs (Mast Laboratories Ltd.), which were then firmly applied to the agar surface.

By varying the size of the bacterial inoculum, the lower limit of assay could be altered. If fewer bacteria were inoculated, the sensitivity was greater. However, with the lowest numbers of bacteria it was difficult to measure the resultant zones of inhibition since individual colonies at the periphery of the inhibited zone were widely separated. For those test organisms used, the ideal inoculum for each 100 ml agar solution lay between 0.05 ml and 0.5 ml of an overnight culture in nutrient broth.

Prior to incubation the plates were left at room temperature for 2 hours so that pre-diffusion of antibiotic could take place. All plates were read after incubation for 18 hours*, by which time clearly distinguishable bacterial growth was present, except in the zones around the antibiotic containing wells (Fig 3:1). Table 3:1 shows the test bacteria and cultural conditions found to be most suitable for the antibiotics used in this study. It also shows the lowest concentration of each drug which could be measured.

In /

* With Sarcina lutea as test organism around 30 hours incubation is required.

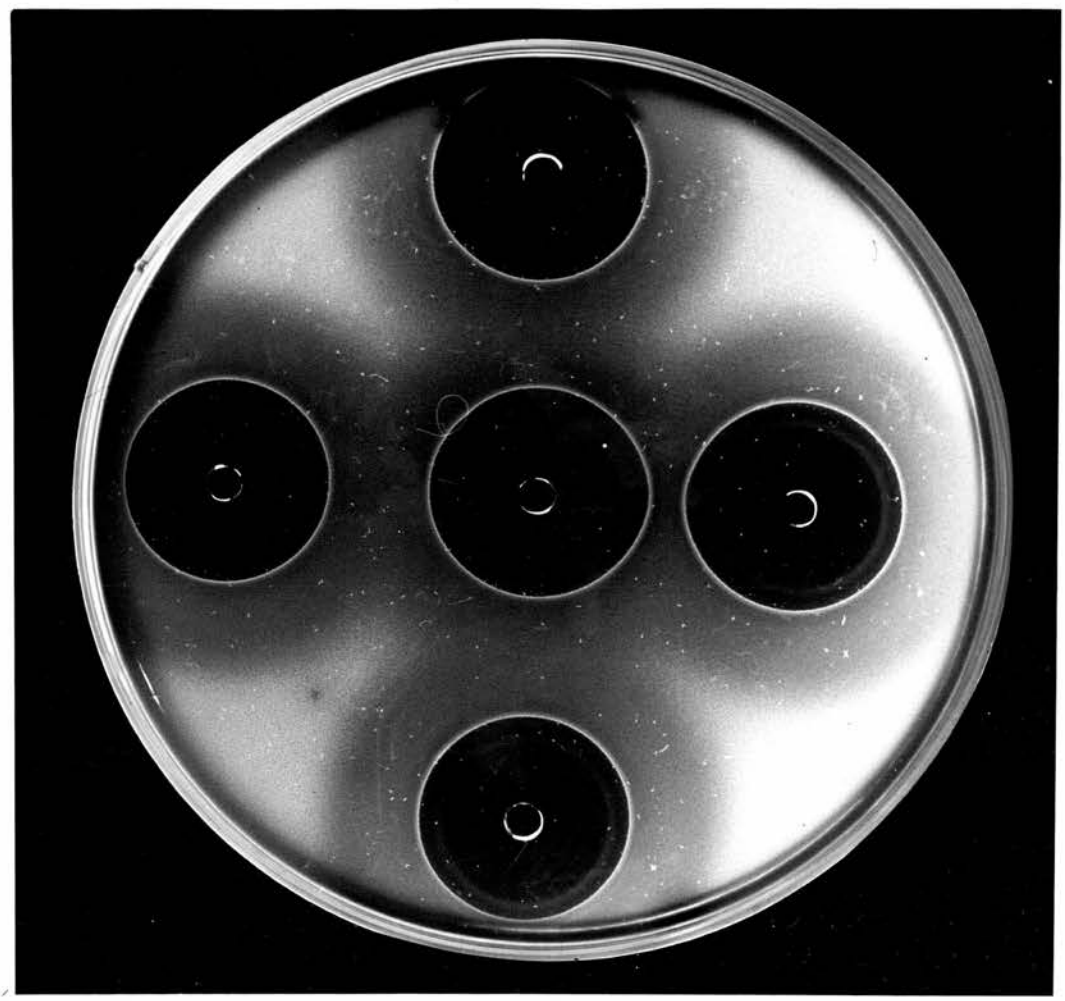


Figure 3:1

Typical assay plate to show the degree of growth and the zones of inhibition produced by ampicillin added to 4 mm diameter wells. (Sarcina lutea)

Table 3:1

ORGANISMS USED TO ASSAY EACH ANTIBIOTIC AND BASIC ASSAY DATA

Antibiotic	Assay Organism	pH	Incubation temp. °C	Lower limit of assay (mg/l)
Ampicillin	Sarcina lutea	6.5	37	0.03
Cloxacillin	" "	6.5	37	0.1
Penicillin	" "	6.5	37	0.05
Cephalexin	" "	6.5	28	0.2
Clindamycin	" "	7.4	37	0.2
Fucidin	Corynebacterium xerosis	6.0	37	0.4
Gentamicin	Serratia marcescens	8.0	37	0.39
Tobramycin	Escherichia coli	8.0	37	0.39
Mecillinam	Sarcina lutea	6.5	28	0.39

In the studies of inflammatory exudate from skin windows, the discs obtained from the experimental lesions were applied direct to the prepared assay plates and compared with standards prepared in pooled normal human serum (P.N.S.) applied via identical filter paper discs. (Fig 3:2). Concomitantly, blood samples were taken and the separated sera were assayed and compared with appropriate standards.

ANALYSIS OF ASSAY RESULTS

Whenever possible, each standard or test solution was set up in quadruplicate and the mean zone diameter was calculated from the four individual results. When the standard concentrations and the resultant zone sizes were plotted, the curve produced fitted a polynomial equation of the type:-

$\bar{y} = a + bx + cx^2$ (Bennett et al, 1966) where

x is the coded concentration (see * below)

y /

* If X mg/l is the lowest concentration, coded result is 1
If the concentration is 2 X mg/l the coded result is 2
If " " " 4 X mg/l " " " " 3
If " " " 8 X mg/l " " " " 4
If " " " 16 X mg/l " " " " 5

Figure 3:2 Assay plate showing a large zone of inhibition around a 12 mm filter paper disc impregnated with 3.1 mg/l of clindamycin. (Sarcina lutea)

y is the mean zone diameter at that concentration and

a, b and c are constants for a given set of assay conditions (the test concentration must fall between the highest and lowest standards)

The equation can be solved for a, b and c using each set of standards and then a, b, c and the mean zone diameter (Y) for an unknown sample can be substituted in the expression:-

$$x = \frac{(b^2 - 4ac + 4cY) - b}{2c}$$

to give the coded value of the unknown concentration. Then the unknown concentration (T) is calculated:-

$$T = \text{lowest standard concentration} \times \frac{\text{antilog } (0.301 \times)}{2}$$

This method has been applied by a simple 'on line' computer programme using BASIC language (Table 3:2 - programme data, Table 3:3 operational demonstration of the programme).

The reproducibility of the overall assay procedure described here using four tests per sample is within 10%. However, for the exudate samples where only one test could be set up each time, the reproducibility would only be within 25%.

In /

Table 3:2

COMPUTER PROGRAMME FOR ANALYSIS OF ANTIBIOTIC ASSAYS (BASIC LANGUAGE)

```
1   Print "Bioassay by the Method of Bennett et al"
2   Print "Enter concentration of strongest standard
        solution"
3   Input Z
4   Print"Enter standard diameters smallest first
        commas in between"
5   Input Y(1), Y(2), Y(3), Y(4), Y(5)
6   For 1 =1TO 5
7   Let T = T + Y(1)
8   Let S = S + Y(1)*Y(1)
9   Next 1
10  Let B = (2*Y(5) + Y(4) - Y(2) - 2*Y(1) )/10
11  Let C = (2*Y(5) - Y(4) -2*Y(3) - Y(2) + 2*Y(1) )/14
12  Let A = T/5 - 2*C
13  Let S=S - T*T/5
14  Let V = B*B*10 + C*C*14
15  Let R = (S-V)/2
16  Print "Regression coefficients"
17  Print A, B, C
18  Print "Residual mean square"
19  Print R
20  Print "Enter test diameter"
21  Input D
22  Let X = (SQR (B*B-4*C* (A-D) ) -B)/(2*C)-2
23  Let W = Z*2↑X
24  Print "Test concentration=", W
25  End
```

Table 3:3

DEMONSTRATION OF ASSAY ANALYSIS

User number ---- E285

Password

Ready

Old:Assay

ANTIBIOTIC - TOBRAMYCIN

Ready

Run

Assay 16:22 Sysed Tue. 29th Mar. 1976

Bioassay by the Method of Bennett et al

Enter concentration of strongest standard solution

? 48

Enter standard diameters smallest first commas in between ? 11.68, 16.62, 20.96, 24.86, 28.21

Regression coefficients

20.9831 4.13 - .258571

Residual mean square

6.45725E-4

Enter test diameter

? 20.98

Test concentration = 11.9937

Running time: 2.6 secs.

In the case of serum gentamicin assays it has been possible to compare the accuracy of results by our method with that of those obtained in other centres of the United Kingdom. (Reeves & Bywater, 1975). This comparison is summarised in Appendix 2.

RESULTS

1. Antibiotic Concentrations in Skin Window Exudates of Normal Subjects

The type of information obtained for each drug is shown in Fig. 3:3. This gives the serum and exudate levels of cloxacillin after an oral dose of 500 mg. Half an hour after the administration of this antibiotic a skin abrasion was made and the exudate in the 3 subsequent hours was collected and assayed. The horizontal dotted lines represent the exudate level (uncorrected for the difference in weight in the exudate discs as compared with the serum standards^{xx}). Figs. 3:4 and 3:5 give comparable studies with cephalexin and fucidin; in the case of the fucidin experiment, duplicate skin windows were made and the reproducibility of the method was thereby tested. Table 3:4 gives the mean concentration of each antibiotic in the hourly exudates from /

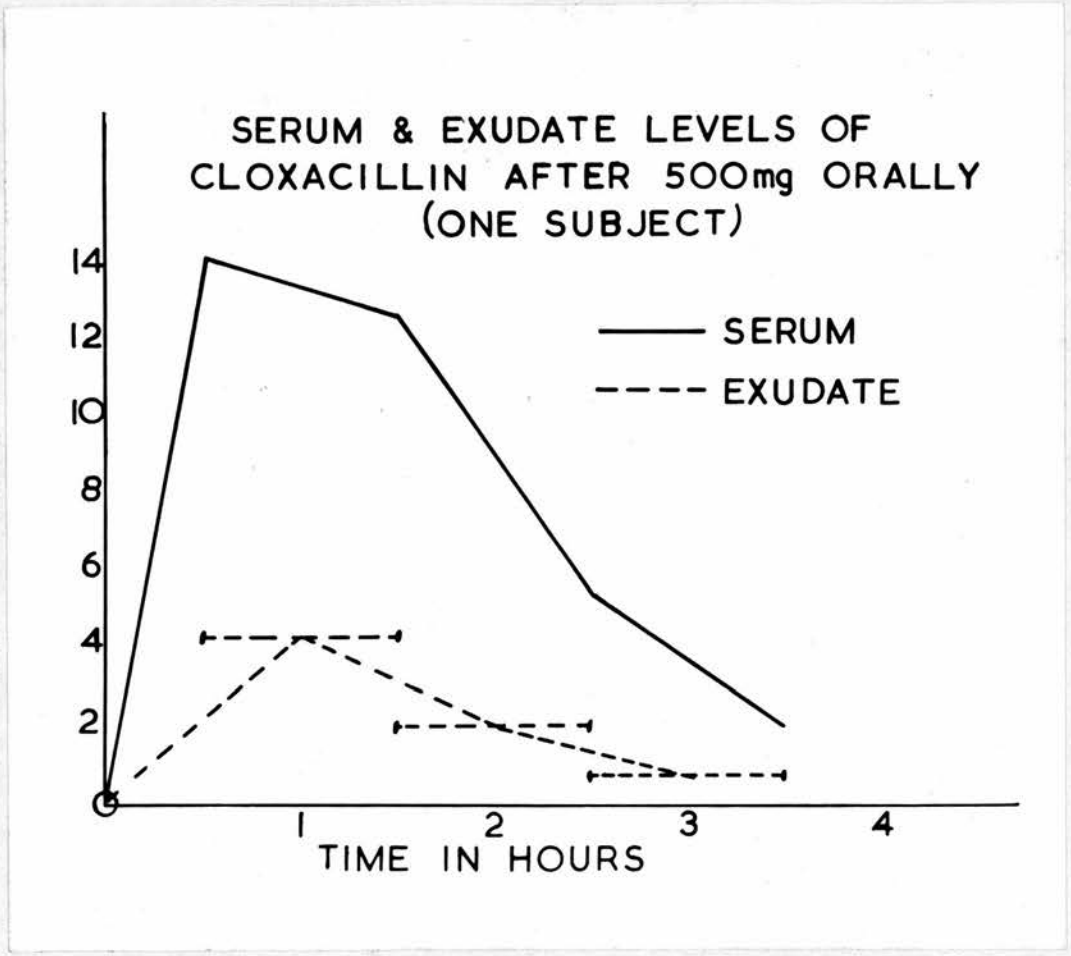


Figure 3:3 Curves showing the serum and exudate levels of cloxacillin following an oral dose of 500 mg at 0 hours.

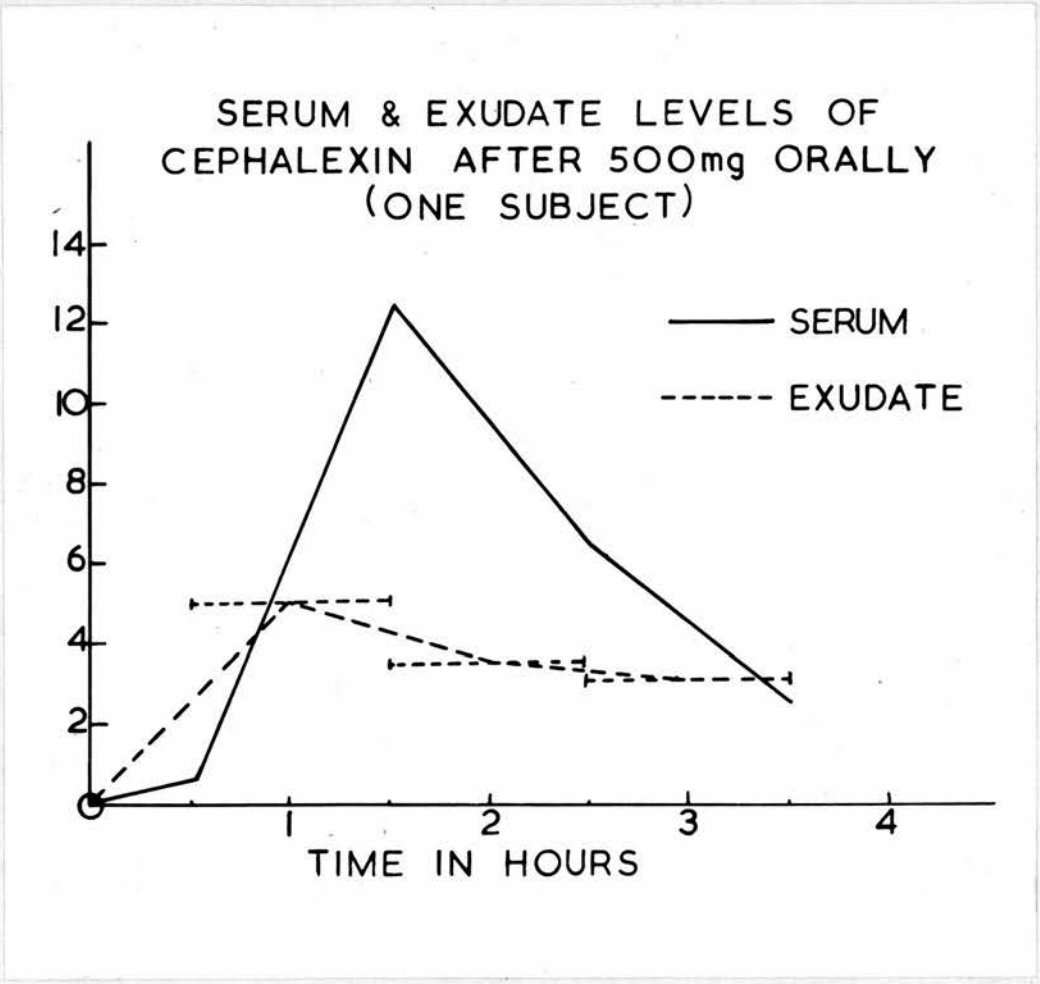


Figure 3:4 Curves showing the serum and exudate levels of cephalexin following an oral dose of 500 mg at 0 hours.

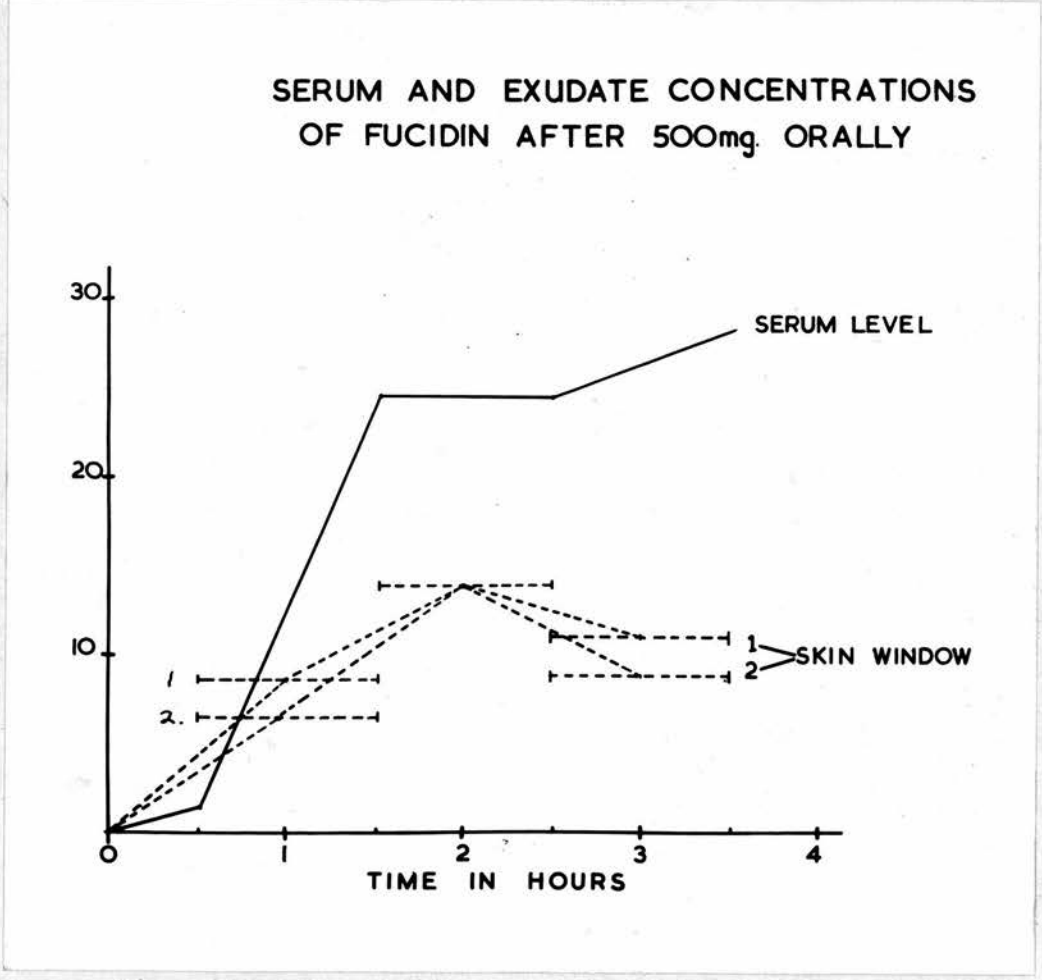


Figure 3:5 Curves showing the serum and exudate levels of fucidin following an oral dose of 500 mg at 0 hours.

Table 3:4

MEAN CONCENTRATION* OF EACH OF 6 ANTIBIOTICS IN INFLAMMATORY EXUDATE

Antibiotic	n	Dose given (mg)xx	1st Hour Conc.*	1st Hour S.E.M.*	2nd Hour Conc.*	2nd Hour S.E.M.*	3rd Hour Conc.*	3rd Hour S.E.M.*
Cephalexin	4	500	2.3	0.85	2.0	0.44	1.0	0.68
Clindamycin	4	300	1.0	0.35	0.6	0.41	0.2	0.2
Cloxacillin	3	500	1.4	1.31	0.7	0.64	0.26	0.26
Fucidin	4	500	3.7	1.49	8.9	1.87	5.3	1.62
Tobramycin	2	50	<0.4	-	<0.4	-	<0.4	-
Mecillinam	2	400	0.78	-	0.79	-	0.50	-

* All concentrations are in mg/l

xx Unless specified in the text, the subjects were studied after a single dose as listed in this table.

from the healthy subjects. Table 3:5 expresses these exudate levels as a percentage of the concomitant serum level. The numbers are too small to make valid comparisons of the antibiotics. However, it is interesting that fucidin, which is 95% proteinbound, achieved the highest sustained proportion of the serum level in the exudate. In contrast cloxacillin, which is also highly proteinbound, (94%) attained only 10% of the serum concentration in the exudate.

2. Skin Window Studies in Subjects with Chronic Lymphatic Leukaemia

Antibiotic kinetic studies were performed on 5 patients with chronic lymphatic leukaemia using fucidin as the test drug. Much lower concentrations were obtained in the exudate from these subjects than from controls. This effect was even more pronounced when the exudate concentration was expressed as a percentage of the concomitant serum level (Table 3:6). Table 3:7 gives the comparative data for exudate weight, serum levels and leucocyte morphology. In the third hour the highest proportion of the serum level (4.9%) was assayed in the exudate of /

Table 3:5

MEAN CONCENTRATIONS OF SIX ANTIBIOTICS IN SKIN WINDOW EXUDATES EXPRESSED AS A PERCENTAGE OF CONCOMITANT SERUM LEVELS x

(Comparison with degree of protein binding)

	Hour of Inflammation			
Antibiotic	1st Hour	2nd Hour	3rd Hour	Protein binding
Cephalexin	38	19	20	25%
Clindamycin	34	9	4.9	70 - 90%
Cloxacillin	10	7	7	94%
Fucidin	42	46	28	95%
Tobramycin	<5.5	<6.1	<8.7	25 - 30%
Mecillinam	26	26	28	10 - 20%

x For dosage see table 3:4

Table 3:6

SKIN WINDOW EXUDATE LEVELS OF FUCIDIN IN PATIENTS WITH CHRONIC LYMPHATIC LEUKAEMIA

(following an oral dose of 500 mg)

			Hour of inflammation					
Subject	Age	Sex	I		II		III	
1	58	F	0*	(0)	1.5*	(3.6)	4.7*	(11.5)
2	63	F	0	(0)	0.2	(3.5)	1.7	(32)
3	68	F	0.5	(1)	2.15	(2)	3.3	(6.1)
4	49	M	0	(0)	1.4	(4)	0.6	(1.4)
5	59	M	0	(0)	0	(0)	1.4	(19)
MEANS			0.1	(0.1)	1.0	(2.6)	2.3	(4.9)
S.E.M.			0.1		0.4		0.7	
Normal subjects								
MEAN			3.7		8.9		5.3	
S.E.M.			1.49		1.87		1.62	

* mg/l (% of serum level)

Table 3:7

WEIGHT OF EXUDATE AND SERUM AND EXUDATE CONCENTRATIONS FROM SKIN WINDOWS IN 5 PATIENTS WITH CHRONIC LYMPHATIC LEUKAEMIA AND 4 NORMAL SUBJECTS[x]

Hour of inflammation	Study Group	Mean weight exudate (mg)	Mean serum level (mg/l)	Mean of exudate/serum %
I	Normal	11.74	8.7	42
	Leukaemic	7.07	13.6	0.1
II	Normal	10.79	19.4	46
	Leukaemic	7.26	38.6	2.6
III	Normal	9.20	18.8	28
	Leukaemic	6.21	47.6	4.9

x Please compare with Table 3:6 (page 72) and the text (page 70)

of the leukaemic patients.

3. Correction Factors

Since there is no mathematical technique for obtaining the correct exudate concentration when zones which use a variable low weight of exudate (approximately 5 - 20 mg) are compared with those using the greater weight of standard solutions (35.6 mg $\pm$ 2.1), correction graphs have been produced for the antibiotics studied to compare the effects of different weights of standard. (Appendix 3). The application of such corrections to some results from table 3:4 shows that the acute inflammatory exudates contain higher antibiotic concentrations than that table indicates. Table 3:8 shows the 'corrected' results for cephalexin, clindamycin and fucidin.

Table 3:8

MEAN EXUDATE CONCENTRATIONS OF THREE ANTIBIOTICS IN NORMAL SUBJECTS FOLLOWING CORRECTION++ FOR EXUDATE WEIGHT

Antibiotic	n	Hour of Inflammation I	II	III
Cephalexin	4	11(117)*	9.4(69)	4.3(44)
Clindamycin	4	3.1(130)	1.2(43)	0.4(18)
Fucidin	4	14.0(160)	26(134)	18(96)

* All concentrations are given as mg/l. Figures in brackets are the exudate level as a percentage of the serum level.

++ Because the exudate weights were usually much less than the weights of antibiotic standards the assay results in Tables 3:4 - 3:7 underestimate the true exudate concentrations. Table 3:8 indicates the highest concentrations likely in the exudate. The antibiotic concentrations studied for anti-staphylococcal effect in Chapter 5 were based on Table 3:4.

DISCUSSION

Studies in Normal Subjects

The concentration of antimicrobial drugs achieved in an individual tissue is of great relevance when that site is infected (Kunin et al, 1973). During the therapy of infection the interstitial fluids and the stroma of the various normal tissues, contain vastly differing concentrations of antibiotics. In contrast the inflammatory response to infection tends to be similar in all tissues. Thus studies of antibiotics in acute inflammatory exudates may provide important information about the efficacy of an antibiotic in any infected tissue. The technique described in this chapter is a new approach to the study of antibiotic kinetics in man (Raeburn, 1971) and it provides an opportunity to study host responses to infection (chapters 2 & 4) as well. However, far more normal subjects must be studied if comparisons between antibiotics are to be possible.

Recently Tan et al, (1972) have used a skin window method to examine antibiotic levels in tissue fluids and have concluded that drugs with a high degree /

degree of protein binding attain lower concentrations in such fluids than those which are poorly protein-bound. Their method differs from my own in an important respect for they cover the skin lesions with a chamber containing buffered saline. Thus the antibiotics are studied in an additional (unnatural) body compartment. Since the chamber is filled with non-protein fluid, the proteins of the inflammatory exudate are greatly diluted (to about 1/25 of their initial concentration). Thus one would anticipate that chamber fluid would equilibrate with the 'free' antibiotic. Furthermore, the one in 25 dilution must affect the accuracy of the initial antibiotic assays. Table 3:9 compares the results with cephalexin from this thesis (corrected for the weight of exudate) with those quoted by Tan et al, (1972). It is clear that the two methods provide different but complementary information; my method shows the situation in acute inflammatory exudate, while Tan's technique may indicate the situation in non-inflamed tissue fluids. It is interesting that another technique for examining interstitial fluid antibiotic levels, using subcutaneous teflon chambers (in /

Table 3:9

CEPHALEXIN IN SKIN WINDOW EXUDATES

(CONTRASTS BETWEEN TECHNIQUES)

Time after abrasion (hrs.)	This thesis (corrected for weight)	Tan et al (1972)
0.5	117*	N.T.
1.5	69	4*
2.5	44	30
3.5	N.T.	29
Mean peak serum level	13.8mg/l	14.4mg/l
Mean peak exudate level	11mg/l	3.1mg/l

* Exudate levels as % of concomitant serum levels.

N.T. = Not tested.

(in dogs) provides fluid with a low protein content and also shows higher concentrations after the less protein-bound antibiotics. (Chisholm et al, 1973).

The results here show that if there is a normal inflammatory response, protein-binding may not have an adverse effect. On the contrary some protein-bound drugs may enter the inflamed site via a "piggyback phenomenon". However, the contrast between fucidin and cloxacillin, both highly protein-bound drugs, indicates that protein-binding alone (as measured by conventional methods) is not the only factor which affects inflammatory exudate levels.

The time at which the highest proportions of antibiotic reach the exudate (1st hour of inflammation) is a time when few leucocytes have been mobilised (Chapter 2). However, elsewhere we have found that the exudate of the first few hours is rich in proteins, particularly IgA and IgM (McClelland et al 1974).* Antibiotic may well be protein-bound but not necessarily to albumin.

There are other observations which support the suggestion that protein-binding can sometimes aid antibiotic distribution. Mattie et al, 1973), using /

* This study did not separate the first, second and third hours of inflammation.

using an *in vivo* model in mice, showed that naficillin which is about 90% protein-bound in the mouse had greater therapeutic effect against an experimental staphylococcal infection than did cloxacillin (80% protein-bound in mice). These authors took great care in controlling their experiments so that the only difference between the two groups of experimentally infected animals was the degree of protein-binding. Ellis *et al*, (1975) have compared inflammatory exudate and wound exudate levels of cefazolin (84% protein-bound) and cephaloridine (less than 20% protein-bound) using a disc technique (the inflammatory exudate method was identical to our own). Both antibiotics achieved high levels in inflammatory exudate and there was no difference in the proportion which passed from the serum. Exudates from surgical wounds contained lower concentrations of both antibiotics but the highly protein-bound cefazolin was not at any disadvantage.

It is not yet possible to construct a unifying hypothesis to explain all the findings with antibiotics in inflammatory exudates. Taken as a whole they suggest that it is wrong to assume (as is all too frequent eg Garrod & O'Grady, 1968) that /

that high degrees of protein-binding always impair antibiotic pharmacokinetics.

Studies with Patients who had Chronic Lymphatic Leukaemia

This group of patients were investigated because they had a chronic illness in which infections requiring antibiotic therapy were frequent. On the other hand they were all in a stable clinical state and the minimal trauma necessary for the exudate studies, seemed justified. There were no complications following the skin window abrasions.

The results of these experiments were surprising since they showed that the C.L.L. patients absorbed fucidin well and the serum levels achieved were around twice as high as in normal subjects, (the patients were considerably lighter than the healthy subjects). Despite the higher serum levels, exudate concentrations in the C.L.L. patients were much lower than in controls. Furthermore the exudate/serum relationships at each hour of inflammation were different, so that the course of acute inflammation in the leukaemic subjects appeared /

appeared to be more gradual.

After surveying the literature I have been unable to find any comparable studies with antibiotics in leukaemic subjects. Their abnormal immunological responses are well known (particularly hypogammaglobulinaemia in chronic lymphatic leukaemia) and these appear to explain the frequent infections. If the inflammatory response is more gradual, or is diminished, this might, as indicated here with fucidin affect the localisation of antibiotics in infected sites.

These studies emphasize the importance of host factors during the treatment of an infection. The recovery of a leukaemic patient may not only be hampered by his poor immunological responses; in addition there may be secondary effects such as an interference with antibiotic localisation via the inflammatory response. Clearly this aspect must be investigated further in any group of patients whose immune function is impaired.

SUMMARY OF CHAPTER 3

1. Adequate concentrations of several antibiotics can be demonstrated in the acute inflammatory exudate of the skin.

2. During the first hour of inflammation higher proportions of antibiotic seem to occur in the exudate when compared with serum levels. (During this period there are very few exudate cells).

3. After three hours the antibiotic activity of normal exudates was lower but cell concentrations were increasing.

4. In normal subjects no antibiotic so far studied reached higher proportions in the later exudates.

5. The ability of an antibiotic to penetrate into inflammatory exudate is not solely determined by protein-binding and fucidin, which is 95% protein-bound achieved the highest exudate levels.

6. Patients who had chronic lymphatic leukaemia achieved very low fucidin concentrations in the exudate especially in the first hour of inflammation.

GENERAL /

GENERAL

This Chapter, and the previous one, showed the concentration of certain antibiotics and of phagocytic cells in human inflammatory exudates. The following chapters study *in vitro*, the antibacterial effects that such concentrations of antibiotics or cells might have.

THE ANTIMICROBIAL ACTIVITIES OF PHAGOCYTIC CELLS

CHAPTER 4

THE ANTIMICROBIAL ACTIVITIES OF PHAGOCYTIC CELLS

CHAPTER 4.

"If it were done when 'tis done, then't were well It were done quickly;"

(Macbeth).

INTRODUCTION

The skin window studies of phagocytic cells reported in this thesis and by other authors (Rebuck & Crowley, 1955, Southam & Levine, 1967, Senn et al 1969) emphasize that these cells rapidly accumulate in inflamed sites. Their subsequent function is to phagocytose, and later to kill, a wide spectrum of micro-organisms. The killed organisms are then digested by the action of a variety of lysosomal enzymes. Table 4:1 summarizes these anti-microbial activities of phagocytic cells with a note about the effector systems and the source of energy (based on Karnovsky, 1962, Cohn & Hirsh, 1965, Zeya & Spitznagel, 1966, Baehner & Nathan, 1967, Lahey et al 1974 and Stossel, 1974.

To study these phagocytic functions I have used methods based on the direct action against a specific microorganism /

TABLE 4:1

Anti-bacterial Systems of Neutrophil Granulocytes

System	Intermediates	Energy Source	Site of killing	Comments
Phagocytosis	membrane synthesis	anaerobic	not applicable	reduced outwith temperature range 33 - 41°C.
pH effects	lactate accumulation etc.	glycolysis	phagosome	can fall to pH 3.5
Cationic proteins	electrophoretic fractions I - VIII	-	phagolysosomes & extracellular milieu	Inhibited by protease inhibitors in plasma
Lactoferrin	chelates of iron	-	phagoglysosomes & exterior of phagocytic cell wall	Stored in secondary granules with alkaline phosphatas
Myeloperoxidase (M.P.O.)	H_2O_2 + Halide	glycolysis & H.M.P. activity	phagolysosomes	Main antistaphylococcal system
Superoxide (O_2 -)	NADH or NADPH plus O_2 + oxidases	"	"	O_2 is destroyed by superoxide dismutase
Aldehydes	H_2O_2 /			

TABLE 4:1 contd.

System	Intermediates	Energy Source	Site of killing	Comments.
Aldehydes	H_2O_2 + M.P.O. + chloride } deamination & decarboxylation	HMP pathway	phagolysosomes	Not easily distinguishable from other systems
Lysosomal enzymes				
a) Lysozyme	May need other enzymes or complement	-	Phagolysosomes & extracellular milieu	Attacks mucopeptides of bacterial cell walls
b) Other degradative eg Phospholipases	Agents which increase access to bacterial cell wall	-	phagolysosomes (N.B. mainly degrade killed organisms)	Marked variations between bacterial species.

micro-organism, Staphylococcus type 42b. This is a coagulase positive strain maintained in subculture since 1952 which seems to have low pathogenicity in the human and which is highly sensitive to penicillin (minimum inhibitory concentration is 0.006 mg/l.). The phagocytic and bactericidal activities have been examined in this work but digestion of dead intracellular organisms, not studied here, is also of great importance.

Phagocytosis

This is a rapid process in either granulocytes or blood monocytes and their derivatives. Phagocytosis by these "professional phagocytes" is greatly increased by pre-treatment of the target particles with serum which contains opsonins. In the case of the neutrophil polymorphonuclears some complement factors are also required. Phagocytic activity can be stimulated by a polypeptide substance, tuftsin, which is synthesised in the spleen (Constantopoulis et al 1973). Tuftsin has a direct action on the phagocytic cells, in contrast to opsonic substances which act by coating the bacteria thus increasing their susceptibility to phagocytosis.

Phagocytosis utilises energy derived from anaerobic metabolism. That it does not depend on aerobic pathways has been demonstrated by Karnovsky (1962) and /

and Baehner & Nathan (1967), who showed that with or without aerobic conditions lactate is produced during phagocytosis. Agents which block aerobic pathways do not reduce phagocytosis but inhibitors of glycolysis such as fluoride or iodoacetate produce at least an 80% reduction (Cohn 1970).

Synthesis of cell membrane is required for continued phagocytosis to take place (Werb 1975) and this is reflected by evidence of increased phospholipid content. The ability of granulocytes to synthesise and recycle cell membrane is limited and there is thus a definite restriction of their lifespan and of their capacity for phagocytosis. Monocytes and macrophages derived from blood monocytes, can synthesise and repair the cell membrane and they can engage repeatedly in both pinocytosis and phagocytosis.

The process of phagocytosis is rapid; over 90% of an inoculum containing 5×10^6 staphylococci per ml can be ingested in 15 minutes by granulocytes at the same concentration (van Furth & van Zwet 1973).

Intracellular Killing /

Intracellular Killing

This process is much more difficult to study and the metabolic processes which accompany it are complex and controversial. However, there is general agreement that the final process of a major killing mechanism is the confrontation of intracellular bacteria with hydrogen peroxide, myeloperoxidase and a source of halide which is usually iodide. The source of hydrogen peroxide is still uncertain but it requires an increment of aerobic metabolism via the hexose monophosphate (H.M.P.) shunt. The enzymes crucial to this aspect of intracellular killing are listed in Table 4:2 along with some indication of their main function, (based on Stossel, 1974, Root et al, 1975, Homan-Müller, Weening & Roos, 1975).

Briefly, the energy released from the pentose pathway is utilised via a hydrogen transport system (either NAD/$NADH_2$, NADP/$NADPH_2$ or both) to produce superoxide (O_2-) from oxygen under the influence of oxidase enzymes. Superoxide is itself bactericidal or it reacts with hydrogen peroxide (H_2O_2) to form hydroxyl radicals (and thence aldehydes), which are also antibacterial. Superoxide /

TABLE 4:2

Intracellular enzymes and bacterial killing

Enzyme	Cofactor	Biochemical action
Glucose-6-phosphate dehydrogenase	NADP	Oxidises glucose-6-phosphate
6-phosphogluconate dehydrogenase	NADP	Oxidise 6-phosphogluconate
NADH or NADPH oxidases		Forms O_2 - from O_2
Catalase	-	Removes H_2O_2
Myeloperoxidase	Halide	" " (+ Halogenation mediated killing of bacteria)
Glutathione redutase glutathione peroxidase		Removes H_2O_2
Superoxide dismutase		Converts O_2 - to H_2O_2

Superoxide in aqueous solution can generate H_2O_2 spontaneously and the mixture of these two peroxides is bactericidal for some strains of staphylocccci (Babior, Curnutte & Kipnes, 1975). The hydrogen peroxide generated is available for the myeloperoxidase - mediated bactericidal system. There is much to be learnt about these linked bactericidal systems, particularly about their specificity against different organisms.

Chronic granulomatous disease (C.G.D.) is the prototype of disorders of intracellular killing but there is some controversy as to the defect. Some have shown a reduction of NADH oxidase activity to less than 25% of normal values (Baehner & Karnovsky 1968), while others have not (Holmes & Good 1972). Recent studies have shown that in C.G.D., the levels of superoxide in phagocytosing granulocytes are grossly reduced compared to normals. This still does not identify the primary biochemical lesion but it has been found to be the most promising test, so far available, for recognising female carriers of the X-linked form of C.G.D. (Weening, Wever & Roos, 1975).

Quite /

Quite separate from these controversies which concern mechanisms for generating H_2O_2, there is good evidence that other bactericidal systems exist in phagocytic cells. These are also listed in Table 4:1. Evidently each phagocytic cell has reserve mechanisms available that could compensate for inefficient activity of other pathways.

Intracellular Digestion

Dead bacteria must be removed and this function is served by the group of lysosomal enzymes. Lysosomes, heterogenous as regards both their size and biochemical actions, are easily visible on electron microscopy or with high power light microscopy. Table 4:3 lists some enzymes contained in lysosomes with a note on their subcellular origin, pH optima and likely digestive function. (from Bainton, 1972 & Barrett, 1972). It is relevant that in those genetic diseases in which lysosomal enzymes are known to be deficient, there is seldom the clinical problem of infection (Harkness, 1972). This above all, separates the digestive activities of phagocytic cells from those which accomplish killing.

Table 4:3

SOME ENZYMES OF PRIMARY AND SECONDARY LYSOSOMES

Enzyme	Optimum pH	Cell site	Action
Lysozyme (muramidase)	6.2	1ry lysosomes ($\frac{1}{3}$) 2ndary " ($\frac{2}{3}$)	Hydrolyses part of bacterial cell walls
Lipases	4.2 - 4.5	1ry lysosomes	Hydrolyse glyceryl esters of triglycerides & phospholipids
Cathepsins (A - E)	2.5 - 6.5	1ry lysosomes	Degrade proteins & polypeptides
Other acid hydrolases eg i) B-glucuronidase	4.3	1ry lysosomes & endoplasmic reticulum	Removes terminal residues from oligosaccharides & glucuronides
ii) α-L-fucosidase	5.6 - 6.1	1ry lysosomes	Degrades oligosaccharides
RNAse	5.4 - 6.7	1ry lysosomes	Depolymerizes R.N.A.
Collagenases	4.0	1ry lysosomes	Degrades & solubilizes collagen
Myeloperoxidase	7.4	1ry lysosomes	Removes H_2O_2
Alkaline phosphatase	7.6 - 9.9	2ndary "	Unknown

ASSESSMENT OF PHAGOCYTIC FUNCTIONS

Phagocytosis

A basic technique for the measurement of phagocytosis is to incubate the cells and bacteria together in a culture medium containing serum and to prepare slides so that the intracellular bacteria may be counted (van Furth & van Zwet 1973), (Figs. 4:1 and 4:2). The information obtained is the proportion of cells which are capable of phagocytosis and the mean number of intracellular organisms per phagocytic cell. This method gives no information about the kinetics of phagocytosis or about intracellular killing. However, the assessment of phagocytosis in purified cell preparations, such as monolayers of monocytes on glass, is a useful marker as to cell type (van Furth et al 1972).

Rate of Phagocytosis

Further information such as the speed of phagocytosis can be obtained by sampling a suspension of cells, bacteria and serum during incubation at 37°C. After spinning the aliquots at 100g for 4 min, (to sediment the leucocytes while leaving extracellular bacteria in the supernatant), viable counts /

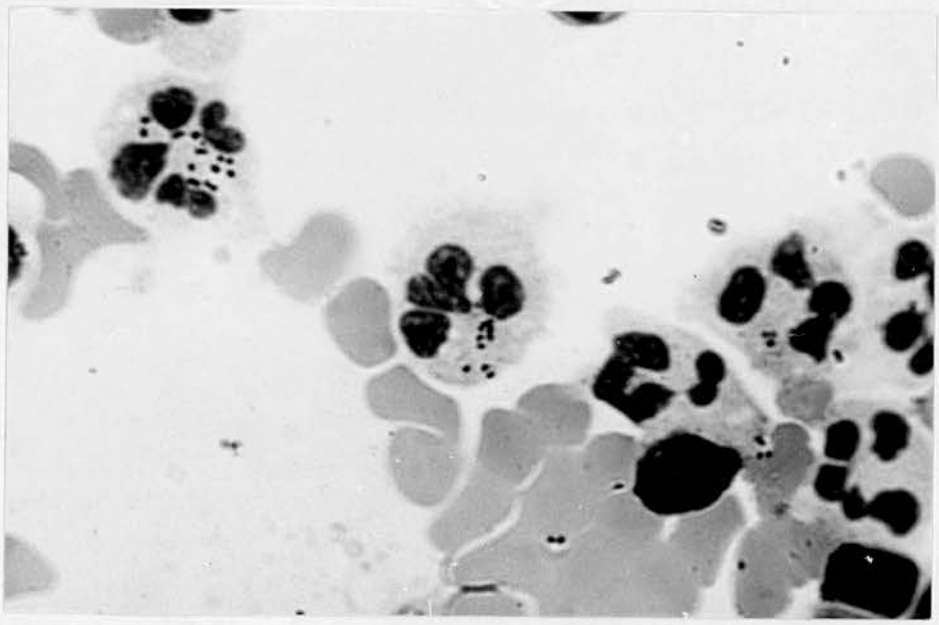


Figure 4:1 High power view to show intracellular staphylococci after ingestion by a neutrophil.

(Giemsa stain x 1000)

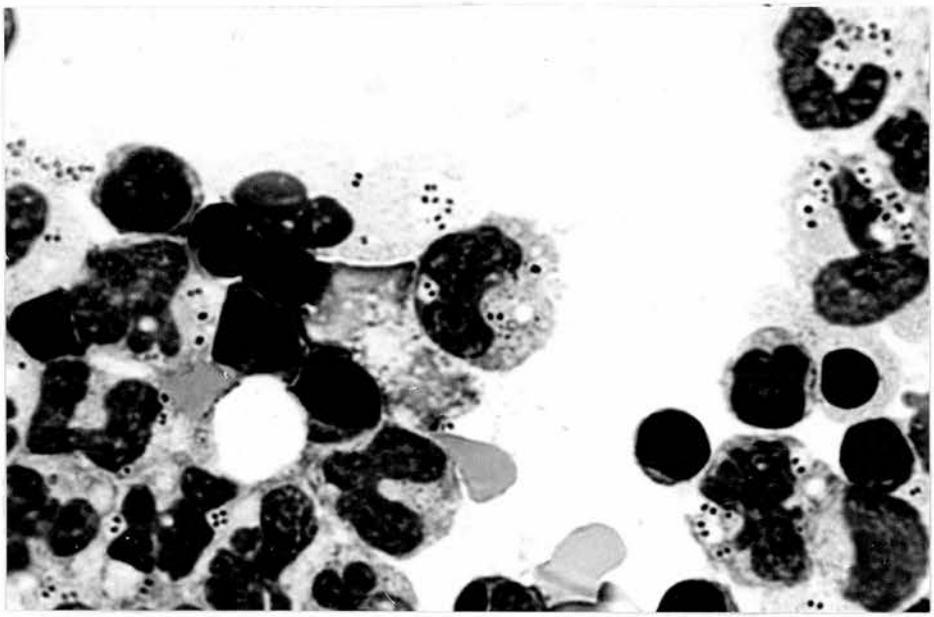


Figure 4:2 High power view to show intracellular staphylococci after ingestion by a monocyte.

(Giemsa stain x 1000)

counts are performed on the supernatant by a pour-plate method (Maaloe 1946). Such studies have shown that normal human granulocytes can phagocytose roughly 91% Staphylococcus aureus in 15 minutes (van Furth & van Zwet 1973) or 90% Pseudomonas pyocyanea in one hour (Young & Armstrong 1972), but only in the presence of serum.

Intracellular Killing

There is considerable variation in techniques to measure intracellular killing. The most exacting method is described in detail by van Furth & van Zwet (1973). Briefly, the phagocytic cells (1×10^7 per ml) are mixed with an (estimated) equal number of the test bacteria plus 10% human AB serum. After a sufficient period of incubation to allow phagocytosis (usually 15 mins) the preparation is spun slowly (100g) thus sedimenting the leucocytes and intracellular bacteria. The cell layer is carefully washed and then re-incubated in fresh medium for 2 hours while sub-sampling regularly. Each sub-sample is diluted (1 in 2) and the cells are sedimented and then lysed with distilled water. After careful agitation the viable bacteria released are counted by a standard pourplate method. Changes in /

in the number of viable intracellular bacteria during incubation, probably represent the killing ability of the phagocytic cell studied. However, an avoidable limitation is the tendency of many bacteria to form clumps so that in the counts 10 organisms or so may only form one colony. Simpler methods are based on the fall in total bacteria (extra and intracellular) in a phagocyte/serum suspension (Biggar et al 1971). Some authors rely on the use of antibiotics, added to the medium after a period of phagocytosis to kill extracellular organisms, (eg. Alexander et al 1968). A variant of this principle utilises lysostaphin an antistaphylococcal enzyme which, while remaining extracellular, rapidly destroys most strains of staphylococci (Baughn & Bonventre, 1975). Solberg (1972) has developed a further method in which the intracellular killing of some aliquots is inhibited in vitro by phenylbutazone, thus making possible an estimation of the killing which occurs in the earliest minutes of incubation. In other methods the initial killing process is obscured by phagocytosis.

A new method which has promise as a screening test uses a completely different principle. Neutrophils (or other phagocytic cells) are incubated with bacteria /

bacteria while adherent to glass as a monolayer. After phagocytosis has occurred the preparations are washed and incubated with ^{3}H - thymidine. This will label the viable bacteria which can be demonstrated by autoradiography. Since dead bacteria will not label, an estimate of intracellular killing is possible. Cline (1973) who developed this technique has claimed that it distinguishes clearly between patients with chronic granulomatous disease and controls. It is not applicable to studies with many species of bacteria such as Escherichia coli. In addition many workers in this field have been unable to repeat Cline's results (van Furth - personal communication).

METHODS IN THIS STUDY

Separation of Phagocytic Cells

1. Mixed leucocytes (used in studies of granulocyte function)

Mixed leucocytes were prepared from heparinised venous blood by dextran sedimentation. Three ml. 5% dextran (M.W. 250,000 - Pharmacia, Uppsala, Sweden) in normal saline were added to 10 ml. blood containing 100 units heparin (without preservatives) and mixed gently before incubation for 30 minutes at 37°C (at a slope of 45°). The supernatant containing leucocytes was then removed and spun at 100 g for 10 minutes. The plasma/dextran supernatant was discarded and the cell button was then washed twice using heparinised physiological saline. The cells were then suspended in Hank's solution (Appendix 4) to a final concentration of 1 x 10^7 phagocytic cells* per ml.

2. Purified monocytes (plus lymphocytes)

These were obtained relatively free from contamination by granulocytes by a method similar to Boyum (1968). Twenty ml. of 45% ficol (Pharmacia, Uppsala, /

* counted in a Neubauer chamber

Uppsala, Sweden) in double distilled water was mixed with five ml. of 39% hypaque, (Nyegard, Oslo, Norway). The mixture was sterilised by filtration through a 0.4 u filter (Millipore UK Ltd.) and 4 ml. aliquots were placed in sterile Falcon tubes (15 x 90 mm). Eight ml. portions of mixed whole blood (containing heparin 10 units/ml.) were carefully layered over the ficol/hypaque columns. After centrifugation at 420 g* for 20 minutes the tubes showed four distinct columns (Fig 4:3). The top layer (plasma plus platelets) was discarded, the second layer contained monocytes and lymphoid cells and the third layer was mainly ficol/hypaque mixture. The granulocytes and clumped erythrocytes formed the bottom layer. For these experiments the second (monocyte) layer was taken, mixed with an equal volume of physiological saline plus 0.001 Molar EDTA and recentrifuged at 100 g. The sedimented cells were washed three more times with saline/EDTA and resuspended in medium 199 to the required cell density.**

* 1800rpm for 'Christ' bench centrifuge and 14 cm. radius for swingout head.

** The proportion of monocytes in this suspension ranged from 12% to 40% with a mean of 28%.

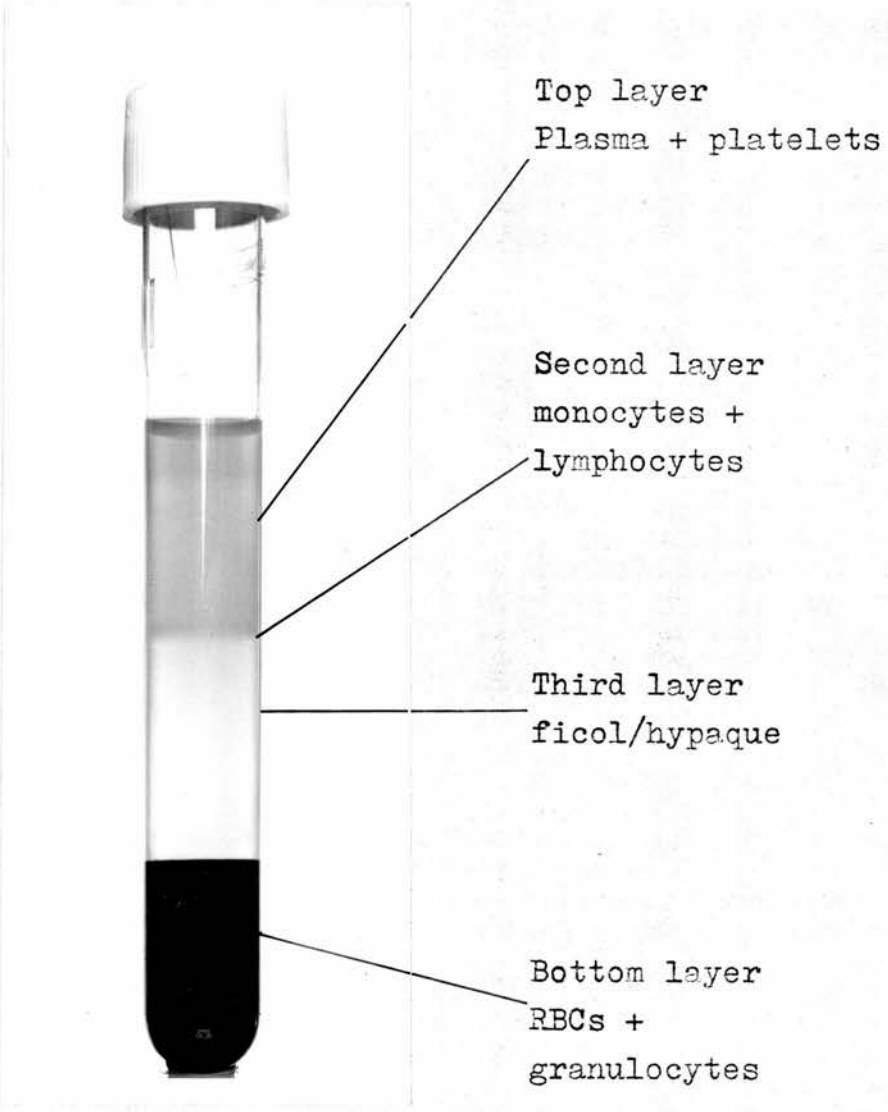


Figure 4:3 Tube showing 4 layers from which monocytes and lymphocytes (second layer) can be separated.

Phagocytosis Techniques

1. Cell monolayers on glass coverslips within Leighton tubes containing 5% CO_2 were incubated at 37°C for an hour with medium 199 containing 10% AB serum and 2 x 10^7 staphylococci per ml. They were then washed six times with warm medium, rapidly air dried and mounted face uppermost prior to staining. After giemsa staining the proportion of cells which were phagocytic and the average number of bacteria per cell could be assessed (Figs. 4:1 & 4:2). This gave only qualitative information.

2. The kinetics of phagocytosis was studied by taking phagocytic cells in a concentration of 1 x 10^7/ml. and mixing with an estimated 1 x 10^7/ml. of staphylococci (type 42b). AB serum was added to a final concentration of 10%. After a control sample had been taken the mixture was incubated (with rotation at 16 rpm) for either 15 or 30 minutes at 37°C. It was then centrifuged at 100 g for 5 minutes, the supernatant was taken for viable counting and the cell button was used for measurement of intracellular killing (see below) or the preparation of smears for microscopy. The unincubated sample was also spun at 100 g and the supernatant/

supernatant was collected. These supernatants were each diluted to 10^{-3}, 10^{-4} and 10^{-5} in saline. Aliquots of 0.1 ml. were then spread on nutrient agar[x] in 9 cm. Petri dishes using bent glass rods. These rods were sterilised between plates by rinsing in methylated spirits and flaming. After overnight incubation at 37°C the number of colonies on each plate was counted using an electric counter which recorded each individual colony when it was touched by a metal probe. Counts of less than ten, or more than 500, colonies per plate were not analysed. The viable count of bacteria in the original supernatant was calculated by finding the mean of duplicate results for each sample after correction for dilution factors. Figs 4:4 and 4:5 summarise the methods used in the quantitative assessment of phagocytosis.

Intracellular Killing

Leucocytes and bacteria were prepared and cultured with AB serum as for phagocytosis testing.[xx] After either 15 or 30 minutes incubation (with rotation) the complete mixture was spun at 100 g for 4 minutes and the supernatant was removed. The cell button was washed twice with heparinised physiological saline and/

x for details please see appendix 4, page 189.

xx The final volume of the cell/bacteria suspension was usually 3.3 ml.

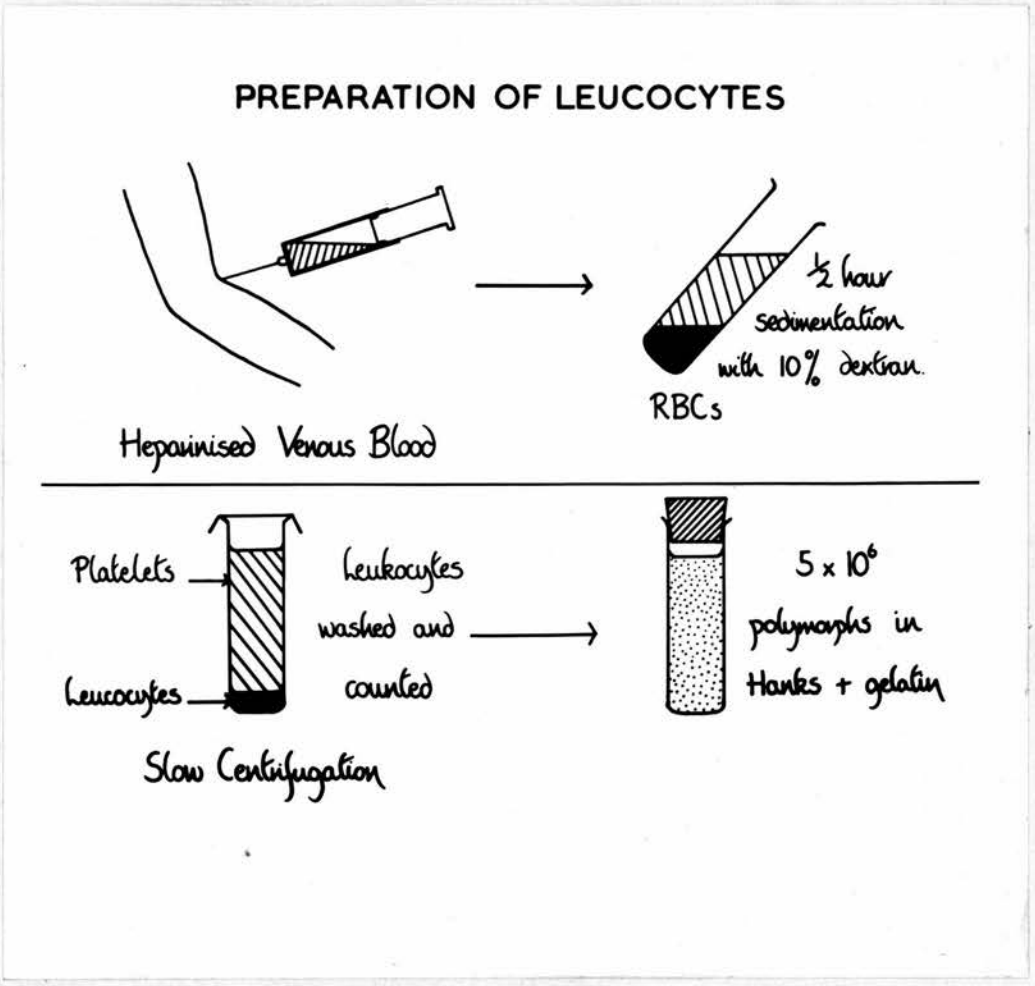


Figure 4:4 Method for separation of leucocytes for studies of phagocytosis and intracellular killing by neutrophils.

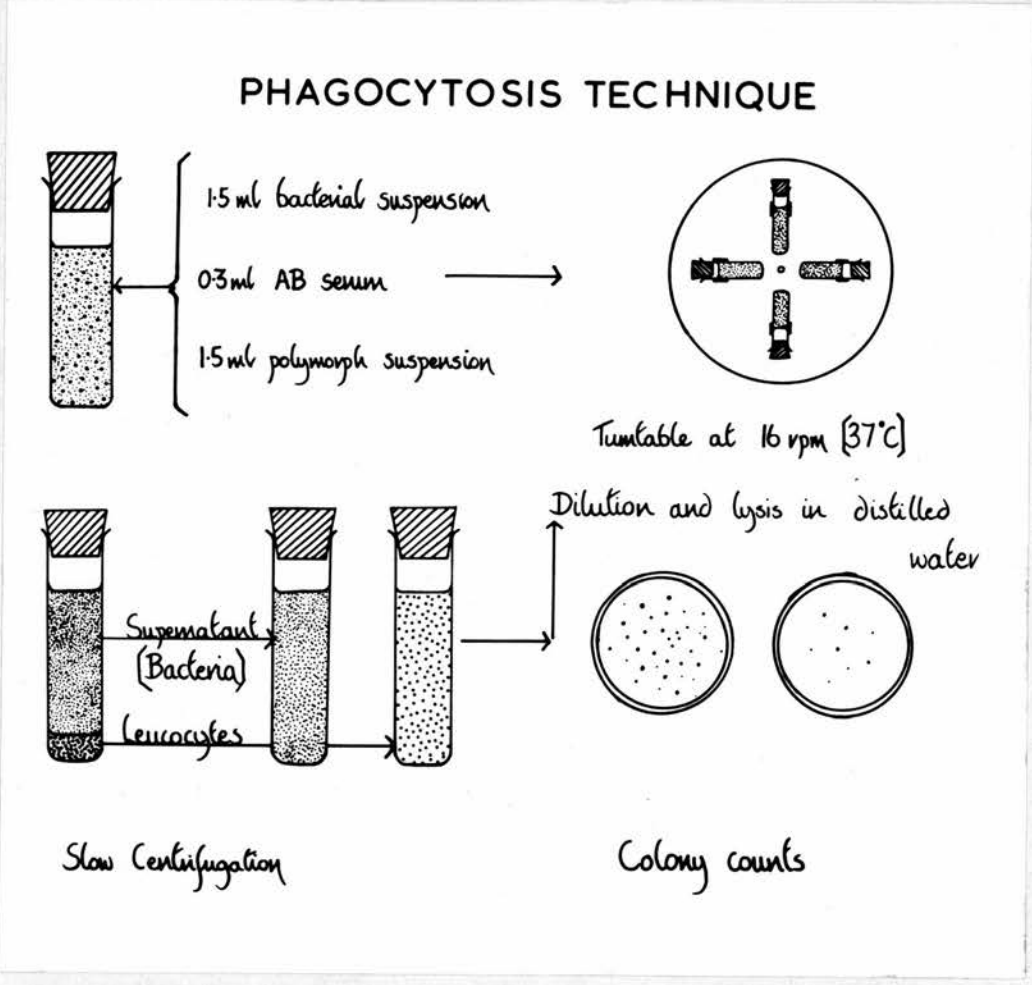


Figure 4:5 Basic technique for measurements of phagocytosis (by counting supernatant bacteria) or intracellular killing (by counting leucocyte associated bacteria).

and then suspended in Hank's solution plus 10% AB serum to the original volume. This mixture was incubated for a further 2 hours with sub-sampling at 0, 30, 60, 90 and 120 minutes. Each 0.4ml sub-sample was mixed with an equal volume of ice-cold Hank's solution, spun at 100g for 4minutes and the supernatant was discarded. The cell button was mixed with sterile distilled water (containing 0.01% bovine serum albumin) and vigorously mixed by pipetting, to lyse the phagocytic cells, thus releasing all bacteria which were still viable. These preparations were diluted in tenfold steps to 10^{-4} and spread on plates for viable counting as detailed on page 107. For most experiments there was a control tube containing bacteria, Hank's solution and 10% AB serum, from which the rate of bacterial growth could be estimated.

STATISTICAL ANALYSIS

1. Phagocytosis (see fig. 4:5)

From the initial extracellular bacterial count (x) and the extracellular count after 30 minutes incubation (y), the phagocytic index at 30 minutes (Ph_{30}) was calculated using the following formula:-

Ph_{30} /

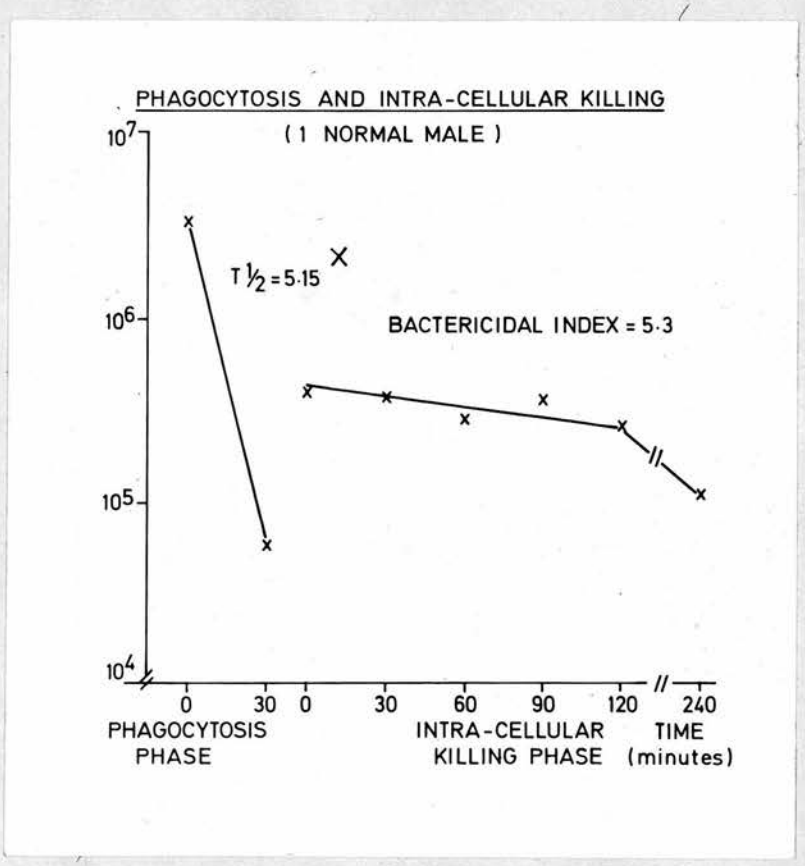


Figure 4:6 Phagocytosis and intracellular killing by neutrophils from a healthy male subject with the calculated $T\frac{1}{2}$ (phagocytosis) and the bactericidal index.

$$Ph_{30} = \log_e x - \log_e y \qquad (i)$$

Thereafter the half time for phagocytosis ($T_{\frac{1}{2}}$) could be computed:-

$$T_{\frac{1}{2}} = \frac{\log 2}{Ph\ 30} \times 30 \qquad (ii)$$

Thus if the initial extracellular bacterial count was 5×10^6 and at 30 minutes it was 1×10^5 then

$$Ph_{30} = \log_e 5 \times 10^6 - \log_e 1 \times 10^5$$
$$= 15.4 - 13.8 = \underline{\underline{1.6}} \quad (i)$$

and the half time for phagocytosis ($T_{\frac{1}{2}}$)

$$= \frac{\log 2}{1.6} \times 30 = 13.0 \text{ minutes} \qquad (ii)$$

2. Intracellular Killing

A typical time course for normal intracellular killing is given in Fig 4:6, which records the number of viable bacteria (n) against time on a semi-logarithmic scale. Since logarithmic transformation produces a near linear relationship with the time of incubation (t), the relationship can be expressed as $\log_e n = a + bt$. Where b is the slope of the relationship and a is the intercept on the y axis, which represents the natural logarithm of the 'idealised' initial count of bacteria. For comparisons between different individuals, the rate of killing is indicated by the slope 'b'. For convenience /

convenience a bactericidal index (B) has been derived from b:-

Bactericidal index (B) = -b x 1,000 (rounded off to 1 decimal place)

This index is high when there is rapid intracellular killing and very low (often less than 1.0) if killing is reduced.

Thus if the number of intracellular bacteria at 0, 30, 60, 90 and 120 minutes was 4×10^6, 1×10^6, 6×10^5, 4.5×10^5 and 2×10^5 respectively then a would be 14.85 and b would be - 0.02267.

The bactericidal index (B) = 22.7, which is at the upper end of the normal range.

When a and b are computed it is easy to calculate the coefficient of correlation (r) for the set of figures and this represents the scatter of individual results[x] from the straight line relationship.

RESULTS

A. Normal Subjects

(i) Phagocytosis & intracellular killing by granulocytes

In the male individual whose results are shown in Fig 4:6 the value for $T\frac{1}{2}$ (phagocytosis, 30 minutes) was/

[x] Differences between the mean results of the $T\frac{1}{2}$ or bactericidal index of the groups studied were tested using the 't' distribution (Armitage P., (1971) Statistical Methods in Medical Research, Blackwell, Oxford)

was 5.15 minutes and the bactericidal index was 5.3. Table 4:4 shows the individual results in 10 healthy females and 10 healthy males. When both sexes are combined the mean $T\frac{1}{2}$ was 5.56 min. ($\pm$ 1.58 = S.D.) and there was no significant difference between the two sexes, 5.67 $\pm$ 1.72 for females and 5.47 $\pm$ 2.22 for males. The mean bactericidal index in 10 females (aged 20 - 70) was 12.73 $\pm$ 5.18 and for 10 males (aged 21 - 31) was 7.21 $\pm$ 3.05. This represents a significant difference between the two sexes ($p<0.01$ using the t distribution). There was no correlation between the $T\frac{1}{2}$ (Phagocytosis) and the bactericidal index in 17 subjects tested by both methods ($r = -0.2804$). The mean age of these healthy controls was 34 years, but the females were older than the males. There was no suggestion in either sex that the bactericidal index varied with age.

A small number of experiments were carried out after a 15 minute period for phagocytosis instead of 30 minutes. These results are shown in table 4:5 and indicate a slight decrease in the $T\frac{1}{2}$ for phagocytosis but an increase in the bactericidal indices. This shows that during the first 30 minutes after ingestion /

Table 4:4

PHAGOCYTOSIS* AND INTRACELLULAR* KILLING OF STAPHYLOCOCCUS 42b BY NEUTROPHILS FROM NORMAL SUBJECTS

Test Code	Age	Sex	$T\frac{1}{2}$ (phagocytosis)		Bactericidal index	
2	23	F	4.65		15.4	
3	42	F	3.9		13.4	
6	56	F	4.95		10.0	
7	58	F	8.64		11.2	
10	54	F	6.03	Mean	6.0	Mean
11	70	F	N.D.	= 5.67	16.8	=12.73
26	26	F	4.1	S.D=1.72	23.2	S.D=5.18
74	22	F	7.79		14.8	
36	36	F	5.3		6.2	
20	20	F	N.D.		10.3	
16	22	M	6.71		8.5	
28	27	M	5.85		12.3	
38	28	M	N.D.		5.1	
40	21	M	5.38		4.9	
50	22	M	3.15	Mean	10.1	Mean
54	30	M	5.15	= 5.47	5.3	= 7.21
69	30	M	4.99	S.D=2.22	6.0	S.D=3.05
102	31	M	7.26		7.2	
106	23	M	7.40		2.4	
116	22	M	3.35		10.3	

Both sexes Mean $T\frac{1}{2}$ = 5.56
S.D. 1.58

* Phagocytic stage = 30 minutes
ND - Not measured.

Table 4:5

PHAGOCYTOSIS* AND INTRACELLULAR KILLING IN NINE HEALTHY SUBJECTS

Test code	Age	Sex	$T\frac{1}{2}$	Bactericidal index
E.L.	26	F	N.D.	40.0
P.D.	18	F	2.44	14.8
M.M.	43	F	4.14	15.0
L.P.	21	F	3.28	16.6
		MEANS	3.29	21.60
		S.E.M.	0.49	6.15
J.A.R.	34	M	3.22	17.5
A.S.	28	M	N.D.	6.2
P.P.	24	M	N.D.	19.0
D.V.	31	M	N.D.	12.4
K.H.	6	M	4.23	15.7
		MEANS	3.73	14.16
		S.E.M.	-	2.27

* Phagocytic stage = 15 minutes
Please compare with table 4:4

ingestion the killing is more rapid than subsequently and that during a 30 minute period for phagocytosis some significant intracellular killing takes place.

Each of the paediatric patients in the infected group or the cystic fibrosis group could not be matched with a healthy child of the same age. Table 4:6 gives the results in 6 healthy children aged 2 to 10. There was no gross difference between this small group and the healthy adults, but a difference between the sexes was not found.

(ii) Phagocytosis by monocytes

Results of five experiments using the monocyte preparations are shown on table 4:7 which compares the $T\frac{1}{2}$ calculated from the extracellular bacteria at either 30 minutes or 60 minutes. The $T\frac{1}{2}$ is considerably longer than that for granulocytes indicating a slower rate of phagocytosis by the monocytes.

B. Subjects with a History of Repeated Infection

(i) Patient with chronic granulomatous disease.

At the time of this study the patient R. McG. was age 6 and had a history of repeated infections since age 2. These involved the chest, cervical lymph glands /

Table 4:7

GRANULOCYTE FUNCTION IN NORMAL CHILDREN [x]

Patient	Sex	Age	$T\frac{1}{2}$	Bactericidal index	
1	M	2	4.14	7.2	
2	F	4	6.18	2.4	
3	M	4	6.02	10.0	Mean 8.27
4	M	6	9.03	8.1	S.D. 4.39
5	F	8	5.32	6.3	
6	F	10	5.81	15.6	

x Details as in table 4:4 (page 115), please see text page 117.

Table 4:7

PHAGOCYTOSIS BY MONOCYTES*

Test No.	Initial monocytes (per ml.)	Bacteria at 0 min. (per ml.)	$T\frac{1}{2}$ (30)	$T\frac{1}{2}$ (60)
M1	1.3×10^6	4.8×10^5	14.11	17.90
M2	4.9×10^6	7.6×10^5	39.67	40.19
M3	4.4×10^6	4.0×10^5	18.50	13.41
M4	5.6×10^6	5.3×10^5	19.26	21.9
M5	9.7×10^6	6.0×10^5	28.60	23.21
		MEANS	24.01	23.32
		S.D.	10.21	10.18

* Monocytes obtained by method 2 (page 103) were mixed with Staphylococcus albus and 10% AB serum in Hank's/gelatine. $T\frac{1}{2}$ was calculated as on page 110 using the 0 min. count and either the bacterial count at 30 minutes ($T\frac{1}{2}$ (30)) or at 60 ($T\frac{1}{2}$ (60)).

glands and the abdomen and the histological features were of a granulomatous reaction. The child had received an 18 month course of antituberculous therapy without benefit. A male sibling had died at six months because of acute bronchopulmonary infection, and the mother had biochemical evidence (quantitative N.B.T. test, Dr. A.D. Bain) that she was a carrier. Three studies of phagocytosis and killing in this boy, his mother and his sister are summarised in Table 4:8 which shows the $T\frac{1}{2}$ (Phagocytosis) and bactericidal indices both before and after various treatments.

(ii) Other patients with repeated infection

These patients all had a history of chronic infection persisting for at least 6 months. The site of the infection and further details including the phagocytic and bactericidal indices are given in Table 4:9. Comparison of the group of infected females and the normal females shows a significant difference ($p < 0.01$) in that the infected individuals had reduced bactericidal indices. There was also a significant difference between the infected males and controls but only at the 5% level. The mean bactericidal index for infected females did not differ significantly from that for infected males.

The /

Table 4:8

GRANULOCYTE FUNCTION IN CHRONIC GRANULOMATOUS DISEASE

(Conditions & methods as in Table 4:4)
(see family tree appendix 5, page 190)

	TEST	TREATMENT	$T\frac{1}{2}$	BACT. INDEX
A.	RESULTS IN RMcG, aged 6			
	1	Nil	8.73	0
	2	Co-trimoxazole	8.54	0
	3	Clofazamine* (in vitro)	15.1	0
	4	Clofazamine**(in vivo)	10.75	3.1
B.	MOTHER OF RMcG			
	1	Nil	3.9	13.4
	2	Nil	3.68	15.0
C.	SISTER OF RMcG			
	1	Nil	3.36	4.5
	2	Nil	4.14	3.9

* 1 mg/l final concentration

** 100 mg daily orally for 3 weeks

Table 4:9

PHAGOCYTOSIS* & INTRACELLULAR KILLING IO INFECTED PATIENTS.

Patient code	Sex	Age	Site of infection	$T\frac{1}{2}$ (Phago mins.)		Bact. index	
32	F	16	Chronic brucellosis	3.8		6.9	
35	F	43	Renal	7.02	Mean =	6.4	Mean =
37	F	38	Lower urinary tract	5.5	6.60	2.9	3.97
68	F	40	Chest	10.6	S.D. =	1.0	S.D. =
81	F	61	Pelvis	6.24	2.29	2.2	2.36
92	F	16	Chest (Bronchiectasis)	6.44		4.4	
23	M	3	Chest	3.1		3.6	
24	M	18	Skin	4.2	Mean =	5.0	Mean =
27	M	$2\frac{1}{2}$	Chest	6.4	4.95	1.1	2.9
34	M	4	Chest	6.1	S.D. = 1.57	1.9	S.D. = 1.75

* Phagocytic stage = 30 minutes.

The infected patients showed no significant difference from controls as regards the $T\frac{1}{2}$ phagocytosis.

(iii) Patients with Respiratory Infection due to Cystic Fibrosis

Clinical details of these subjects are given in Table 4:10. The mean bactericidal indices of both the male and female groups is also different from the appropriate normals but this is only significantly different at the 5% level. The cystic fibrosis patients do not significantly differ from the infected patients in either phagocytosis or bactericidal indices.

C. Reproducibility of bactericidal test in normals and in disease

Since many in vitro conditions can alter the intracellular killing one would expect considerable variation in results from one day to another. Three control patients, two infected patients, two carriers of chronic granulomatous disease (C.G.D.) and two patients with cystic fibrosis had the test repeated after an interval of at least one week. The results are shown in Table 4:11 which lists the two results for each subject and the basic clinical information.

The/

Table 4:10

PHAGOCYTOSIS* & INTRACELLULAR KILLING IN 10 PATIENTS WITH CYSTIC FIBROSIS

(All had repeated chest infection)

Code	Sex	Age	Infecting organism	$T\frac{1}{2}$ (Phago)		Bact. Index	
29	F	23	Staphylococcus	4.86		1.1	
52	F	8	Pseudomonas	7.13	Mean	11.7	Mean
53	F	16	Staphylococcus	5.16	=7.27	6.1	=7.26
91	F	8	Pseudomonas	4.09	S.D. =3.92	3.3	S.D. =3.11
100	F	7	Staphylococcus	14.00		8.5	
51	M	11	Pseudomonas	5.80	Mean	5.2	Mean
55	M	11	"	6.57	=6.62	1.7	=3.87
88	M	11	"	8.46	S.D.	4.2	S.D.
89	M	13	"	7.39	=1.39	7.2	=2.51
93	M	1	Staphylococcus	4.86		1.1	

* Phagocytic stage = 30 minutes.

Table 4:11

RESULTS OF REPEAT BACTERICIDAL TESTS IN 7 SUBJECTS [x]

Codes	Sex	Age	Condition	Bactericidal index 1st test	Bactericidal index 2nd test
29/45	F	23	Cystic fibrosis	5.98	5.42
53/113	F	16	" "	6.01	7.6
24/25	M	18	Skin infection	5.0	3.8
58/81	F	61	Pelvic infection	2.2	2.2
3/121	F	48	Obligate carrier of C.G.D .	13.4	15.0
122/152	F	19	Probable carrier of C.G.D.	4.5	3.9
2/74	F	23	Healthy	14.8	15.4
16/50	M	22	"	10.1	8.5
54/69	M	30	"	5.3	6.0
			Mean	7.48	7.53
			S.D.	4.29	4.76

r = +0.9728

The measurement of bacterial killing within polymorphonuclear leucocytes is clearly liable to many technical errors but the reproducibility in these initial tests is acceptable.

An important aspect of these in vitro studies with staphylococci and granulocytes is the necessity for careful control studies. Thus in initial studies I found that, in the presence of 10% AB serum, but without leucocytes, the staphylococci could multiply about eight-fold within 2 hours. Likewise it was shown that if rotation was omitted there was negligible phatocytosis after 30 minutes. The importance of these and of other factors (such as the bactericidal action of Hank's solution if gelatine or bovine serum albumin are not added), have been stressed by several authors (Cohn and Morse, 1959, Rabinovitch, 1968, van Furth & van Zwet, 1973).

DISCUSSION

Phagocytic cells play a major part in the response to invading micro-organisms and in the past ten years there have been a spate of papers which describe methods for testing granulocyte and monocyte function (see van Furth and van Zwet 1973). It is usually impossible to compare the results of different techniques quantitatively but a body of information has been built up about the qualitative phagocytic cell defects which occur in a wide range of conditions (eg Raeburn 1975). In any situation the rate of phagocytosis and of intracellular killing will differ for each organism tested. In many patients these rates will also show considerable fluctuation from day to day.

The method for assessing phagocytosis and intracellular killing used in this study was very nearly the same as methods described by three other authors. (Li, Mudd & Kapral 1963, Johnston & Baehner 1970, van Furth & van Zwet 1973). In particular, all used strains of Staphylococcus aureus in an initial concentration between 5 and 10 x 10^6 organisms per ml. From their figures one can calculate the $T\frac{1}{2}$ phagocytosis and bactericidal indices and these are /

are compared in Table 4:12. The comparisons, although incomplete because the other authors only quoted mean results or one "typical" experiment, show that our measurements of phagocytosis gave broadly similar findings.* The rate of intracellular killing measured in this work was less than that found by the other authors. The difference between our results and those of Professor van Furth stem in large part from the longer period of phagocytosis (30 minutes) used here. Although this allowed a greater amount of ingestion, it meant that the early, most rapid phase of intracellular killing was obscured. Thus the experiments using a 15 minute period for phagocytosis gave much closer results to those obtained in Leiden. (See table 4:5)

To the best of my knowledge a difference between the intracellular killing of females and of males has not previously been demonstrated. Few authors have stated the sex of their healthy control subjects and when the sex was recorded individual results are not quoted. Our male and female subjects are not identical in all other respects so this observation requires further substantiation. Nevertheless, the results are in keeping with clinical evidence that

males /

* The shorter $T\frac{1}{2}$ in my own studies has not been adequately explained but it could be attributable to different concentrations of opsonins in the AB sera which were utilised.

Table 4:12

COMPARISON OF $T\frac{1}{2}$ & BACTERICIDAL INDICES USING HUMAN GRANULOCYTES & STAPHYLOCOCCUS AUREUS

Study	$T\frac{1}{2}$ (mins)		Bactericidal index	
	*	**	*	**
Li et al., 1963	8.23		12.42	
Johnston & Baehner, 1970	Not done		44.53	
van Furth & van Zwet, 1973	11.25		32.90	
This study, 1976				
Females	3.73	5.57	21.60	12.73
Males			14.16	7.21

* Phagocytic stage = 15 minutes

** Phagocytic stage = 30 minutes.

males suffer more infections than females and with reports that concentrations of immunoglobulin M are one third higher in females than in males (Grundbacher, 1972). For the present it is prudent to ensure that control subjects are sex-matched in addition to other matching, especially when testing for carriers of chronic granulomatous disease.

The studies with human monocytes show that these cells phagocytose Staphylococcus albus at a slower rate than do neutrophils (Table 4:7). This confirms a recent report which showed that the phagocytic defect occurred with Salmonella, E. Coli and Listeria as well as Staphylococcus aureus (Steigbigel et al, 1974). These authors also show that intracellular killing of staphylococci by both neutrophils and monocytes is similar, having corrected for the differences in phagocytosis.

It is well established that tests of intracellular killing can detect the defect present in chronic granulomatous disease. The method of analysis used here helps to quantify the defects and in a proven case repeated tests showed a consistently low bactericidal index. We studied the effect of clofazamine, a possible immunostimulant drug, on this patient's neutrophil /

neutrophil functions and showed a slight reduction of phagocytosis and a minimal improvement of intracellular killing. (Table 4:8). If we analyse the data of Johnston & Baehner (1970) using the same mathematical techniques, the addition of glucose oxidase coated on to latex particles improved the bactericidal index in one C.G.D. patient from 4.8 to 11.4. Table 4:8 also illustrates a pitfall in the investigation of this disease, for the patient's mother, who is an obligate carrier (see pedigree, appendix 5), had normal bactericidal indices on two occasions. In contrast the patient's sister had bactericidal indices which are quite compatible with her being a carrier.

These experiments (Tables 4:4 and 4:9) show that the bactericidal index in a group of patients suffering from a range of infections, in different sites, is significantly lower than in healthy controls. This finding has also been reported by Solberg & Hellum (1972), who showed that the bactericidal defects were probably the result of infection and were associated with prominent cytoplasmic vacuolisation. The low bactericidal indices in cystic fibrosis patients (Table 4:10) in whom the immediate cause of infection is stasis in the respiratory tract, also point /

point to infection as the main causative factor in the neutrophil dysfunction. The cause of this phenomenon is not yet certain but the bactericidal defects may be the result of known metabolic alterations in the "toxic"neutrophils, (McCall et al, 1973). How these occur is also obscure; since neutrophils do not recirculate after reaching a site of infection, the triggering factors must be in the serum.

In experiments with dogs who had induced cellulitis and abscesses Alexander and his colleagues could not show a neutrophil defect even though 5 out of 6 of their patients with surgical infections had mild to moderate decreases in the rate of intracellular killing. (Alexander, Hegg & Altemeier, 1968). In the human the transient neutrophil dysfunction may well play a part in perpetuating a chronic infection. In addition the findings show that if a patient suspected of having C.G.D. is to be properly tested an infected patient should be included as an additional control.

There are many technical difficulties inherent in bactericidal tests as described here and far greater experience in healthy subjects is required. It /

It is better to take aliquots frequently during the intracellular killing phase and the five sampling periods up to 120 minutes used here should perhaps be a minimum. By using the slope of the regression of the natural logarithm of the bacterial count against time as a basis for comparisons, a meaningful average based on the 5 sub-samples is obtained. This will minimize the error produced by variations in bacterial counts in individual samples. For this reason the method has advantages over other statistical techniques which rely on measurements at only 2 time periods (eg Hoffman & Bullock, 1973). Despite these improved techniques, the range of results in normal subjects is wide and it is difficult to interpret a slightly low bactericidal index in an individual patient. The grouped data is of much more significance and by further consecutive studies of patients during an infection and its treatment it may be possible to reassess our conventional concepts and the approach to management.

SUMMARY

A method for testing the action of separated neutrophils or monocytes against a laboratory strain of Staphylococcus aureus in vitro has been exploited in both normal and infected subjects. A concentration of neutrophils which can be achieved in inflammatory sites in vivo (see chapter 2) can ingest a similar number of staphylococci and the mean half-time of this reaction is 5.56 mins (± 1.58 = S.D.). In contrast the half-time for phagocytosis by normal monocytes is 24.01 mins (± 10.21). Intracellular killing by neutrophils is slower than phagocytosis and the half-times measured here in normal subjects range from 29 minutes to 280 minutes. (the intracellular killing studies have been compared using a 'bactericidal index' to avoid any confusion with the rate of phagocytosis. This index is mathematically related to the half-time). Compared with other similar studies these results show rather slower intracellular killing and in part this is because my method concentrates on the "residual killing capacity" which does not take account of any intracellular killing in the first 30 minutes after the start of phagocytosis. Normal females appear to have /

have more rapid intracellular killing than do normal males and this requires further study.

Patients with chronic infections, as a group, have a reduction in intracellular killing capacity without any change in phagocytic rate and this also occurs in patients with respiratory infection due to cystic fibrosis. These findings may well be of clinical significance but at the least they indicate some difficulties in interpreting tests of neutrophil function in individual patients. A simple method for analysing similar studies has been discussed and it makes possible a semi-quantitative comparison between different reports.

THE ANTIMICROBIAL ACTIVITIES OF ANTIBIOTICS IN VITRO AND IN VIVO

CHAPTER 5

THE ANTIMICROBIAL ACTIVITIES OF ANTIBIOTICS IN VITRO AND IN VIVO

CHAPTER 5

"I do not know what our soldiers do to the enemy, but, my God, they frighten me"
(Wellington, 1814).

INTRODUCTION

The approach to this thesis was based on an assumption; this was that the efficacy of an antibiotic treatment could not be assessed fully, unless the important host responses to infection were recognised and measured. If an individual infection is caused by a specific number of bacteria, recovery will occur when these organisms have been destroyed partly by antibiotics and partly by the host defenses. When two antibiotics are compared, the host defences in each treatment group should be similar if differences in the outcome are ascribed to the therapy. Large scale antibiotic trials would tend to reduce major differences in host defences between treatment groups and identify the antibiotic with the highest overall /

overall efficacy. However, individual patients may sometimes benefit from treatment with antibiotics which have not been exceptional in large clinical trials.

From the data in Chapter 2 we can calculate the rate at which the phagocytic cells accumulate in an inflamed site and their approximate concentration. In Chapter 4 the action of phagocytic cells against a single organism, Staphylococcus type 42b, was assayed. In the following studies several antibiotics in concentrations which occur in inflammatory exudate (Chapter 3) were tested against the same staphylococcus in a similar in vitro system.

1. BACTERIAL KILLING BY ANTIBIOTICS IN VITRO

METHODS:

Each drug was prepared in Hank's solution + 0.1% gelatin and then mixed with a suspension of approximately 1×10^7 staphylococci per ml and with 10% AB serum. The final antibiotic concentrations were as detailed in Table 5:1. The mixtures were incubated at 37°C for 4 hours and aliquots were taken at 0, 30, 60, 90, 120 and 240 minutes. These samples /

Table 5:1

KILLING OF STAPHYLOCOCCUS 42b BY FIVE ANTIBIOTICS

(Comparison with $T\frac{1}{2}$ phagocytosis)

Antibiotic	Concentration (mg/l) [x]	$T\frac{1}{2}$ A/B 120	M.I.C. (mg/l) [xx]
Clindamycin	5	187	0.8
Cloxacillin	5	72	0.2
Cephalexin	5	60	0.03
Fucidin	20	>300	0.5
Tobramycin	1	9.7	1.3

		Normals	Infected Patients
Mean $T\frac{1}{2}$ phagocytosis (30 mins)	(From table 4:4)	5.6	5.9

samples were immediately mixed with an equal volume of ice-cold Hank's solution, and were diluted in tenfold steps prior to plating (see page 107). From the resultant counts of viable bacteria, the rate of antibiotic killing could be assessed:-

$$T\frac{1}{2}\ A/B = \frac{\log 2}{\log n_o - \log n_t} \times t\ *$$

Alternatively an antibiotic killing index could be calculated:-

$$A/B\ \text{index} = \frac{\log n_o - \log n_t}{t} \times 1000\ *$$

(for both equations n_o = initial count of bacteria and n_t = count at time t).

RESULTS

Figure 5:1 shows the decrease of viable bacteria in the presence of clindamycin (1 mg/l.) during 4 hours of incubation. Similar curves occurred with cloxacillin and cephalexin, except that with these two antibiotics there was an initial increase in bacterial count at 30 minutes before subsequent killing.

Fucidin/

* The $T\frac{1}{2}$ A/B and the A/B index provide measures of the rate of antibiotic action which can be directly compared with the $T\frac{1}{2}$ (phagocytosis) or the bactericidal index respectively.

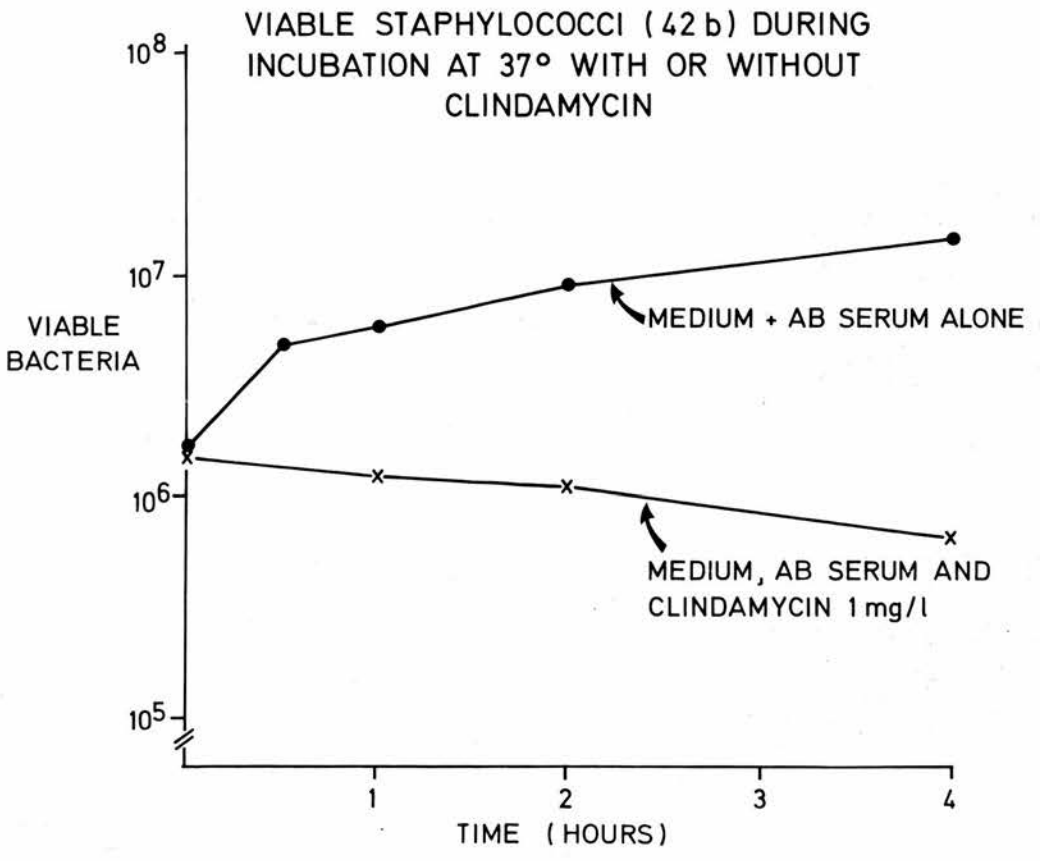


Figure 5:1 Effect of clindamycin (1mg/l. final concentration) upon the number of viable staphylococci in medium + 10% AB serum but without leucocytes.

Fucidin in a concentration of 20 mg/l scarcely reduced the number of viable bacteria ($T\frac{1}{2} > 300$ mins). In the absence of antibiotic (eg Fig 5:1) there was approximately a 12 fold increase in bacteria during 4 hours incubation in this culture system. The mean $T\frac{1}{2}$ A/B for each drug studied is shown in Table 5:1 which also records the minimum inhibitory concentrations (M.I.C.) of each drug[x] measured in nutrient broth by a standard method. (Cruickshank, 1975). The foot note to this table shows the pooled results for $T\frac{1}{2}$ phagocytosis in normal and infected subjects as detailed in Chapter 4. Table 5:2 shows the antibiotic killing indices calculated for each drug with a footnote showing the bactericidal indices already described in Chapter 4.

DISCUSSION

In this cell-free culture system, staphylococcus is slowly killed by some antibiotics but only after an initial delay. Fucidin inhibits growth of the organism but after four hours there was a minimal killing effect. Comparison of the half-times for antibiotic killing of the organism with the half-time for phagocytosis by neutrophils shows that phagocytosis occurs much more rapidly, with the possible /

[x] against staph 42 b

Table 5:2

KILLING OF STAPHYLOCOCCUS 42b BY FIVE ANTIBIOTICS

(Comparison with bactericidal index)

Antibiotic	Concentration mg/l	Antibiotic killing index	S.E.M.
Clindamycin	5	3.7	1.57
Cloxacillin	5	9.6	2.48
Cephalexin	5	11.6	2.98
Fucidin	20	0.2	0.75
Tobramycin	1	71.5	19.0

Bactericidal Indices

	Mean	
Normal males	7.2	0.96
Normal females	12.7	1.64
Infected males	2.9	0.88
Infected females	4.0	0.96
Patient with Chronic Granulomatous Disease	0	-

possible exception of tobramycin. This is very important since it suggests that, in vivo, the majority of bacteria will be intracellular before these antibiotics have acted. Unless the antibiotic enters the cell with the bacteria or can diffuse freely into the cell, it has little opportunity to destroy even such sensitive strains as staphylococcus 42b.

There are no systematic studies of the cell-associated levels of these five antibiotics. Saggers & Lawson (1970) found about 2% of the extracellular concentration of cloxacillin associated with lymphocytes. Alexander and Good (1968) and Mandell (1973) concluded that most antibiotics could not enter phagocytes since they could not show effects on intracellular bacteria. Our own studies, which utilised micro-methods to assay antibiotic in mixed leucocyte preparations[x] (Raeburn et al, 1976), show a different picture (Table 5:3). The important difference is that we assayed the cell concentrations directly, without washing, since this must remove some cell-associated antibiotic. Even allowing for some contamination of the cell layer by supernatant, our findings suggest that more ampicillin and tobramycin penetrate leucocytes than /

[x] see page 212

Table 5:3

MIXED LEUCOCYTE LEVELS OF AMPICILLIN AND TOBRAMYCIN

Incubation time (mins)	Cell concentration in mg/l (% supernatant)	
	AMPICILLIN	TOBRAMYCIN
0	1.9 (67)	0.7 (22)
30	2.3 (74)	1.9 (62)
60	2.4 (80)	2.1 (71)
120	1.7 (61)	1.0 (35)
240	2.0 (66)	0.8 (24)

x Details of the technique are in appendix 6, page 213.

than has been hitherto suggested.

Comparisons of the antibiotic killing indices and the bactericidal indices from normal subjects show that two of the antibiotics tested, clindamycin and fucidin, are less active against staphylococcus 42b than are normal granulocytes. However, the difference is not large. For patients with chronic granulomatous disease, or those with impaired killing during an infection the antibiotics effect could be of critical value. Such antibiotic activity will be maximal in the extracellular situation, complementary to the intracellular killing within the granulocytes.

Such comparisons of the rates of bacterial killing assume that antibiotics and phagocytic cells do not interact and that their effects are additive. However, phagocytic cells may interfere with antibiotic actions (Ritzerfield, 1974) and vice versa (Forsgren et al, 1974). Possible interactions could either enhance or inhibit the rate of bacterial killing. To examine this aspect the following experiments involved the measurement of phagocytic and bactericidal activities of granulocytes in the presence of antibiotics which were administered either in vitro or in vivo.

BACTERIAL KILLING BY PHAGOCYTIC CELLS PLUS ANTIBIOTICS

METHODS

1. Antibiotics added in vitro

In these experiments ampicillin (5mg/l, final concentration) was added to the leucocyte/bacteria mixture in normal clinical dosages after the 30 minute period for phagocytosis and the washing stage. The killing assay was then performed in the usual way[x]. For each experiment a control cell/bacteria mixture was incubated without antibiotic. In one further test the ampicillin was inactivated using penicillinase for 5 minutes prior to lysis of the cell button. (Table 5:4)

2. Antibiotics administered to human subjects

i) Measurements of phagocytosis and intracellular killing were performed on 5 patients undergoing treatment for a chronic respiratory infection. Each patient was roughly matched with a comparable individual who was not receiving antibiotic therapy. Clinical details and the results are shown in Table 5:5.

ii) Measurement of phagocytosis and intracellular killing was performed serially in 6 patients/

[x] See appendix 6 page 218.

Table 5:4

AMPICILLIN AND INTRACELLULAR KILLING

BY GRANULOCYTES

(*In vitro* effects alone and combined)

Experiment	Test Mixture	Bactericidal index	S.E.M.
1	a) Normal cells	14.1	1.9
	b) Normal cells + ampicillin*	12.6	2.6
	c) Normal cells + ampicillin* (+ penicillinase)	14.2	3.8
2	a) Normal cells	11.3	3.3
	b) Normal cells + ampicillin*	9.0	1.2
3	a) Normal cells	37.0	4.1
	b) Normal cells + ampicillin*	30.9	3.6
	c) Ampicillin alone*	25.8	6.0

* Final concentration in mixture 5 mg/l.

Table 5:5

PHAGOCYTOSIS & KILLING IN ANTIBIOTIC TREATED PATIENTS AND MATCHED * (UNTREATED) CONTROLS [X]

Diagnosis	Sex	Antibiotic	Age	$T\frac{1}{2}$	Bactericidal index
Chest infection	F	Penicillin	73	3.8	2.1
Chest infection	F	Nil	72	4.0	4.0
Chest infection	M	Septrin	4	4.1	1.0
Chest infection + candidiasis	M	Nil	4	6.0	10.0
Chest infection + asthma	M	Ampicillin	58	9.5	10.0
Chest infection C.L.L.	F	Nil	60	6.5	16.0
Chest infection	F	Ampicillin	40	10.6	3.2
Behcets synd. + chest infection	M	Nil	44	7.0	6.2
Cystic fibrosis + chest infection	M	Ampicillin	12	5.6	4.3
Cystic fibrosis + chest infection	F	Nil	15	5.2	10.0

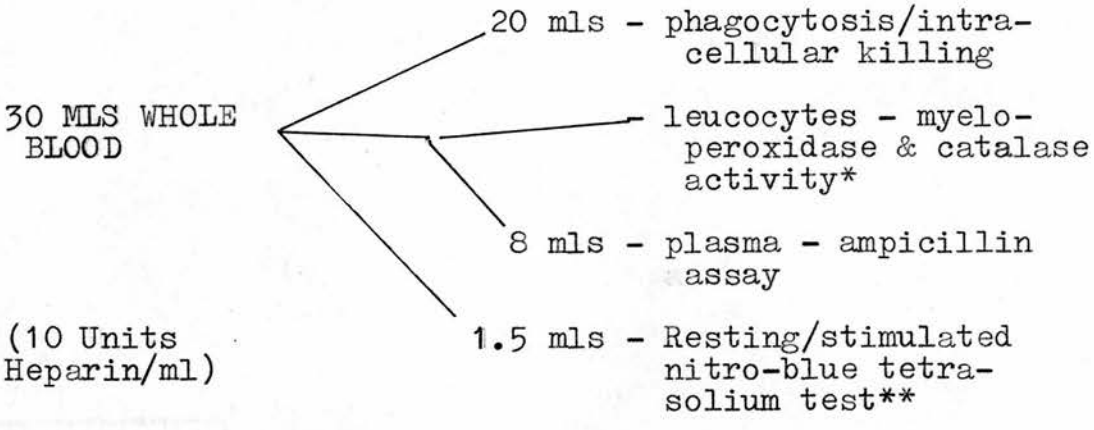
[X] Conditions and methods as in Table 4:4

GROUP	Mean $T\frac{1}{2}$	S.E.M.	Mean Bact. Index	S.E.M.
Antibiotic treated	6.7	1.4	4.1	1.6
Untreated	5.7	0.5	9.2	2.1

* In these preliminary experiments the patients in the treated or untreated groups were not all matched for sex (see Chapter 4, Table 4:4).

patients who were either on or off antibiotic therapy. The clinical information and the results are in Table 5:6.

iii) Four healthy volunteers received ampicillin (500 mg) orally after a control blood sample had been taken. Further blood samples were taken at 1,2 and 3 hours after this single dose. Each sample was immediately heparinised (final heparin concentration was 10 units per ml.) and subdivided as follows for various cell or plasma assays:-



* Performed by Mrs. Marjory Grant & Dr. Angus Harkness

** Performed by Dr. Kenneth Watson

x for methods see appendix 6, pages 213 - 215.

Table 5:6

COMPARISON OF $T\frac{1}{2}$ PHAGOCYTOSIS AND KILLING INDICES WHILE ON OR OFF ANTIBIOTIC

Diagnosis	Sex	Age	$T\frac{1}{2}$ ON	$T\frac{1}{2}$ OFF	INDEX ON	INDEX OFF	Antibiotic (when on)
Chest infection renal failure	F	73	4.0	6.7	4.6	12.2	Penicillin
Brucellosis, + urinary infection	F	14	5.0	N.D.	27.6	6.9	Ampicillin
Cystic fibrosis + chest infection	F	23	5.4	6.0	0	6.7	Clindamycin
Pelvic infection	F	68	6.2	7.5	4.9	2.3	Fucidin
Cystic fibrosis + chest infection	F	16	3.7	5.2	0.8	7.3	Cloxacillin
Chest infection	M	58	9.5	4.9	10.0	16.6	Ampicillin

RESULTS

Tables 5:4 - 9 and figures 5:2 & 5:3 summarise the results of these studies of the combined action of antibiotics and phagocytic cells.

When ampicillin was added *in vitro* there was no significant alteration in the intracellular killing activity (Fig 5:2 and table 5:4).

Table 5:5 shows the clinical details in five pairs of patients who were either on or off antibiotics at the time of the test. These patients were not completely matched and the differences in bactericidal index have not been statistically analysed. However, those receiving antibiotics tended to have lower bactericidal indices.

The patients who were tested twice, first during antibiotic therapy and then when treatment had been discontinued, tended to have lower bactericidal indices (table 5:6). During treatment one individual had a higher bactericidal index while receiving ampicillin but she had a variety of complex medical problems.[x] All the other patients had lower indices when receiving the antibiotic.

These initial studies pointed to a possible influence /

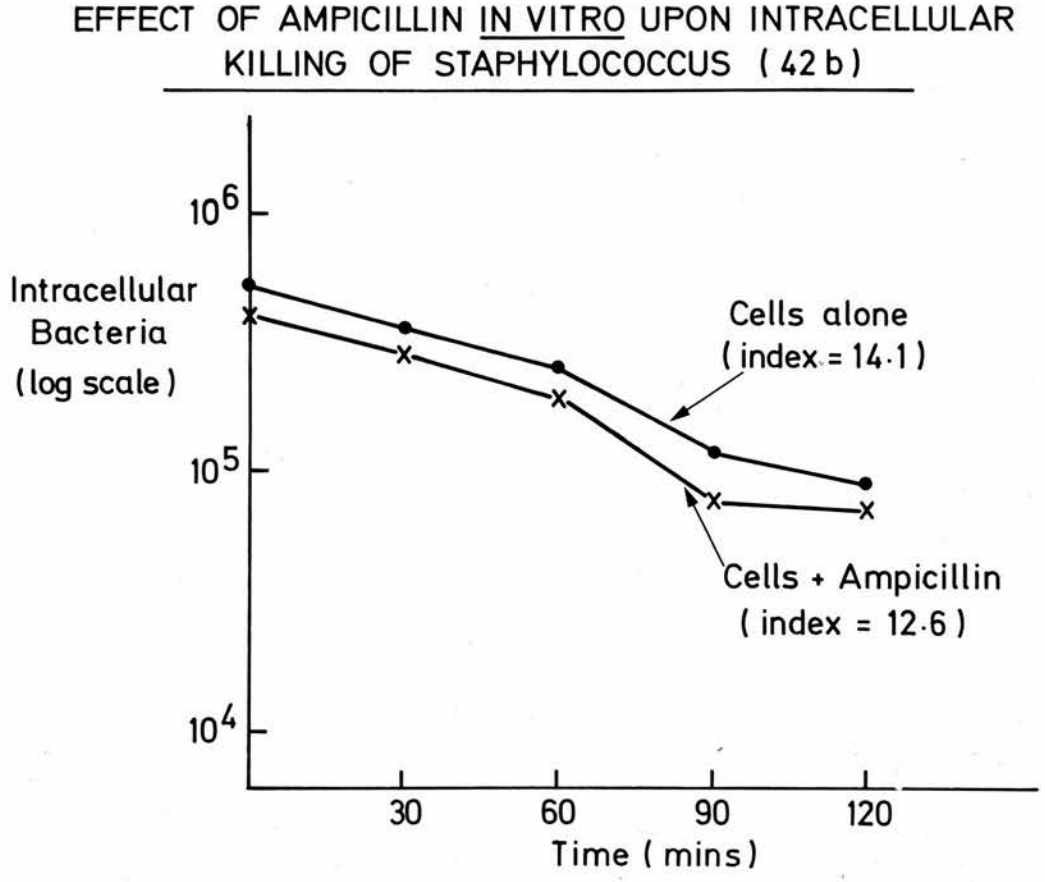


Figure 5:2 The effect of ampicillin (5mg/l final concentration) upon intracellular killing by granulocytes.

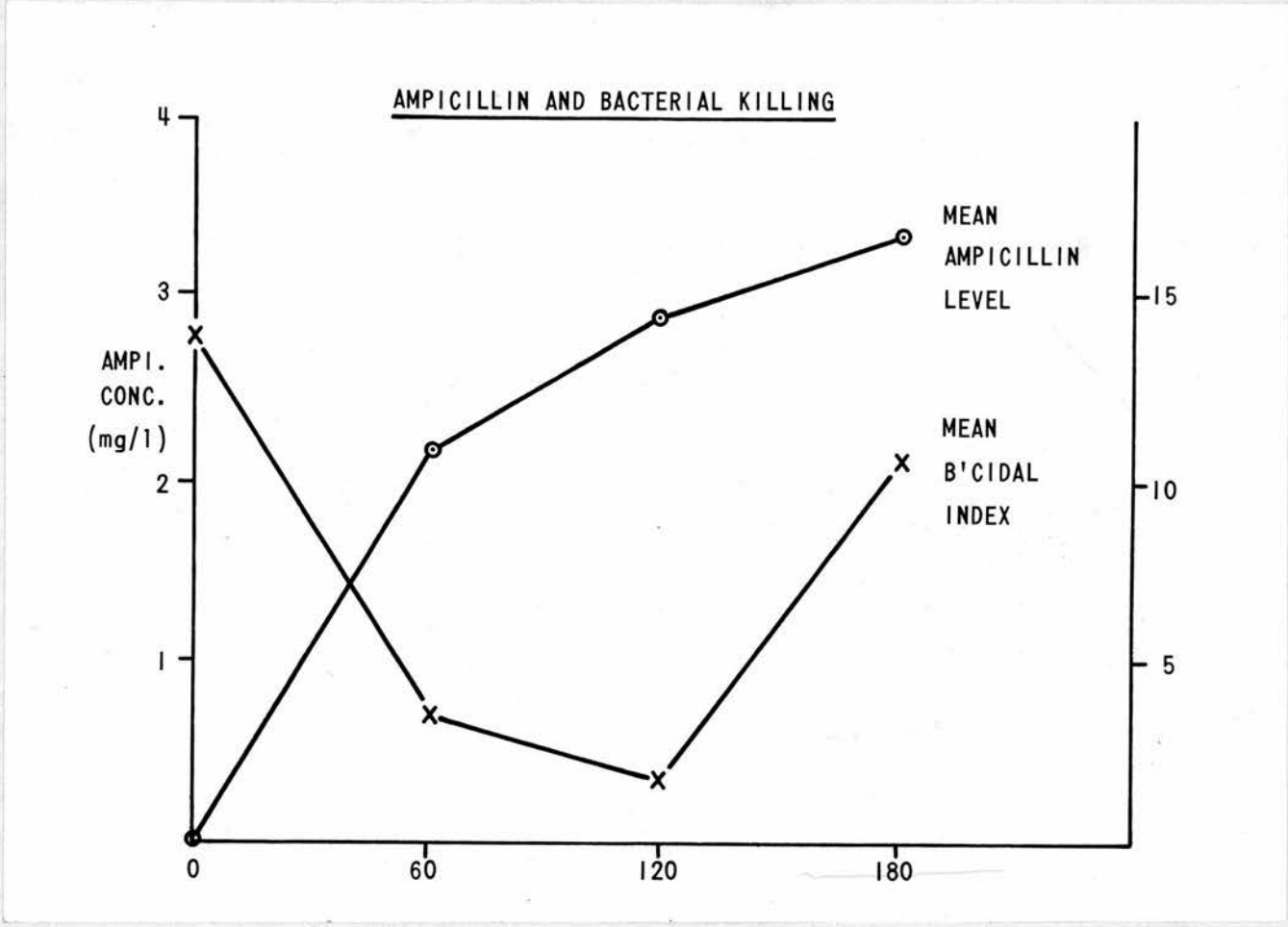


Figure 5:3 Mean bactericidal index (right hand scale) in 3 healthy subjects at various times after oral administration of ampicillin. (Mean serum levels left hand scale). ×

influence of several antibiotics given *in vivo*, upon the intracellular killing by granulocytes. To clarify the effect, studies were performed on healthy subjects who received single oral doses of ampicillin. These were supplemented by enzyme assays performed by Mrs. Marjory Grant, Dr. R.A. Harkness and Dr. K.C. Watson.

The mean bactericidal indices measured serially after ampicillin was given *in vivo* to 3 healthy subjects, are shown in Fig 5:3. Table 5:7 shows the individual results in 4 male subjects at each hourly sampling period following ampicillin, and the means. Despite widely differing starting points, all subjects showed a fall in the bactericidal index at 1 and 2 hours followed by a rise at 3 hours towards normal.

The individual results for leucocyte myeloperoxidase activity and the plasma ampicillin levels are in table 5:8. As the ampicillin level rose the leucocyte myeloperoxidase activity fell but there was a tendancy for the 3 hour level to overshoot the control value. Corresponding results for leucocyte catalase activity and for the increments of N.B.T. reduction capacity (produced by *in vitro* stimulation /

Table 5:7

BACTERICIDAL INDICES IN 4 HEALTHY SUBJECTS WHO RECEIVED A SINGLE ORAL DOSE OF AMPICILLIN (500mg)

Subject	Age	Time after dose (Hours) 0	1	2	3
J.A.R.	33	17.5	3.1	0.5	11.8
P.P.	29	19.0	8.1	3.1	16.0
A.S.	27	6.2	0.2	2.0	4.0
D.V.	27	12.4	2.5	3.4	12.6
MEANS		13.8	3.5	2.3	11.1
S.E.M.		2.8	1.7	0.7	2.5
p			<0.05	<0.02	not significant

Table 5:8 (a)

MYELOPEROXIDASE ACTIVITY*(M.P.O. UNITS/MCG. PROTEIN) IN 4 HEALTHY SUBJECTS AFTER 500MG AMPICILLIN

Subject	Time after dose in hours 0	1	2	3
J.A.R.	5.0	3.6	4.4	6.2
P.P.	1.1	0.92	0.92	0.37
A.S.	0.98	0.49	0.51	1.5
D.V.	1.1	0.85	1.61	2.1
MEANS	2.05	1.47	1.70	2.54

(b)

AMPICILLIN LEVELS (MG/L) IN HEALTHY SUBJECTS AFTER 500 MG ORALLY

Subject	Time after dose in hours 0	1	2	3
J.A.R.	0	4.9	4.5	3.3
P.P.	0	1.0	2.0	4.2
A. S.	0	0.8	2.1	2.6
D.V.	0	10.8	30.5	13.4
MEANS	0	4.4	9.8	5.9

* MEASURED BY MRS. M. GRANT & DR. R.A. HARKNESS.

stimulation of the heparinised whole blood samples with Escherichia Coli) are shown in Table 5:9.[x]

DISCUSSION

The antibacterial activities of phagocytic cells and of antibiotics can be measured in vitro and compared. From these separate measurements it seems that the activity of phagocytic cells against staphylococcus 42b is of a greater magnitude than that of most antibiotics studied (Tables 5:1 and 5:2). In the in vitro study using a mixture of granulocytes and ampicillin, (Fig 5:2, Table 5:4) there was no evidence of a change in the bactericidal index produced by that agent. In vivo the situation may be different.

The first indication of an interaction between granulocytes and antibiotics was that the bactericidal index was apparently reduced in those infected patients who received antibiotics compared to those who did not. (Table 5:5). A similar suggestion stems from the repeat assays of phagocytosis and killing in individual patients (Table 5:6). In both instances the granulocyte activity which altered was the intracellular killing rate and/

x for methodology please see appendix 6, page 213.

Table 5:9 (a)

CATALASE ACTIVITY (MOLES x 10^{-13}/g PROTEIN)*
IN 4 HEALTHY SUBJECTS AFTER 500MG AMPICILLIN

Subject	Time (Hours) 0 x	1	2	3
J.A.R.	3.0	4.4	2.4	2.6
P.P.	1.5	3.1	3.7	5.6
A.S.	1.3	2.2	2.4	3.3
D.V.	0.8	1.0	0.9	1.2
MEANS	1.7	2.7	2.4	3.2

Catalase activity was significantly raised in three individuals, particularly at 3 hours after administration of ampicillin

(b)

INCREMENT OF N.B.T. REDUCTION (%)** AFTER
500MG AMPICILLIN IN HEALTHY SUBJECTS

Subject	Time (Hours) 0	1	2	3
J.A.R.	28	16	10	31
P.P.	26	70	22	24
A.S.	13	1	25	23
D.V.	11	N.D.	1	N.D.
MEANS	20	29	15	26

x Coefficient of variation of 2 control samples in each individual = 7%

* MEASURED BY MRS. MARJORY GRANT AND DR. R.A. HARKNESS

** MEASURED BY DR. KENNETH WATSON

and not phagocytosis. However, these findings are based on small numbers of tests in widely varying clinical situations. The obvious bias is that a patient receiving an antibiotic is likely to be 'more infected' than one who is not and infection itself may cause a drop in intracellular killing (Chapter 4, and Solberg & Hellum, 1972).

The sequential tests of bactericidal function in normal individuals who received a single dose of ampicillin were planned so that the effect of infection could be excluded. The hydrogen peroxide - myeloperoxidase - halide system is so important in intracellular killing that the study was conducted in collaboration with colleagues who could examine the different aspects. The individual results for intracellular killing or for each of the enzyme systems, show that oral administration of 500 mg of ampicillin leads to a change in the measured activity. In isolation, the results in each test are of interest but of uncertain significance. Taken as a whole they suggest that ampicillin acts on a series of important and inter-related metabolic pathways of the leucocyte. Thus it has already been shown that penicillins <u>in vitro</u> inhibit peroxidases/

peroxidase of vegetable origin (Renz et al, 1972) and also human myeloperoxidase (Grant & Harkness, 1976). In addition, there have been occasional reports that administration of an antibiotic to an infected patient causes the percentage of N.B.T. positive neutrophils to fall (Rubinstein et al, 1973, Hawkins, 1973), which could reflect diminished hexose monophosphate shunt activity and hence a reduction in available hydrogen peroxide. Both effects would interfere with the myeloperoxidase killing system and when combined one might anticipate reduced intracellular killing, of the degree that has been described (Table 5:7).

The effect on catalase is difficult to explain particularly since the in vitro studies show that in concentrations of 0.1 m.mol/l, ampicillin inhibits this enzyme (Renz et al, 1972). In vivo the concentrations assayed in our test subjects were between 0.01 and 0.03 m.mol/l, and some stimulation may occur at sub-inhibitory concentrations. Whatever the mechanism, an increase in leucocyte catalase would more rapidly destroy hydrogen peroxide and further hamper the myeloperoxidase bactericidal system.

Figure /

Figure 5:3 demonstrates that as the plasma level of ampicillin rises there is a fall in the bactericidal index. At three hours however, the index has risen while the ampicillin levels persist. There are several possible explanations for this phenomenon but the simplest would be that ampicillin has seeped into the phagosomes of the cells after three hours. Mandell (1973) has suggested that most antibiotics, with the exception of rifampicin, are unable to enter phagocytic cells. His detailed studies did not include ampicillin but they suggested that unless an agent is highly lipid soluble (and ampicillin is not) it is unlikely to penetrate the cell membrane. Our studies show that ampicillin can apparently act on myeloperoxidase (situated in the primary lysosomes), on catalase (in cell membranes and other subcellular fractions) and possibly on the hexose mono-phosphate pathway (in the mitochondria of the cell sap). The only convergence of these enzyme systems occurs within the phagolysosome formed following phagocytosis. It is therefore pertinent that there is an effect on intracellular killing. Ampicillin inside phagolysosomes would not itself demonstrate antibacterial activity if the pH at that site were unfavourable (optimal pH for/

for ampicillin's antibacterial action is around 6.5). Intracellular pH is still something of a controversy but Sprick (1956) has suggested that within the phagosome it can fall to between 4.7 and 5.5.

All of these assays are subject to a variety of technical problems. In particular it is difficult to measure the rate of intracellular killing, sequentially, at hourly intervals. It is relevant that the coefficient of correlation between the logarithm of the viable bacteria and the duration of intracellular incubation dropped at the 1, 2 and 3 hour sampling times. Perhaps the presence of antibiotic affects the precision of viable counting. These studies must be repeated in far larger groups and with more precise techniques.

The experiments in this chapter have assumed a very simple model for neutrophil/staphylococcal interaction. After 30 minutes a few percent of the initial inoculum will remain extracellular and some will divide. In addition, some of the neutrophils which ingest the organism will not survive and will eventually release viable bacteria. These two processes will cause there to be a continual low proportion of extracellular bacteria and it is on these that antibiotics will have their principal effect.

SUMMARY

1. In cell-free preparations the rate of bacterial reduction by four out of five antibiotics was much less than the rate at which phagocytes ingest bacteria.

2. Clindamycin, cloxacillin and cephalexin could kill staphylococcus 42b at about the same rate as that of intracellular killing. Fucidin was much less rapid than intracellular killing. Tobramycin was much more rapid than intracellular killing.

3. In mixtures of phagocytic cells and ampicillin *in vitro* the antibiotic did not appear to influence the rate of phagocytosis or of intracellular killing.

4. When certain antibiotics are given to either patients or healthy subjects *in vivo*, there may be a reduction in intracellular killing.

5. This is probably caused by the inhibition of myeloperoxidase and of oxidative enzymes, as well as by effects on catalase.

6. These findings are of considerable clinical importance and require elucidation and confirmation.

NINE RELEVANT QUESTIONS AND CONCLUSIONS

CHAPTER 6

NINE RELEVANT QUESTIONS AND CONCLUSIONS

CHAPTER 6

"--- now to perform a true physician part
and show I am a master of my art,
I will prescribe what diet you should use,
what food you ought to take and what refuse."

(Ovid, c 10 B.C.).

WHY IS INFECTION PREVALENT DESPITE MODERN ANTIBIOTICS?

Since the introduction of antibiotics into clinical practice there has been a dramatic fall in the morbidity and mortality caused by many infections. The principle change has been a lower death rate due to acute infections such as pneumonia, pyogenic meningitis or scarlet fever (Office of Health Economics 1962, 1963), and the use of chemotherapy must have been a major factor (Garrod, 1970). However, for many acute infections, such as diphtheria or whooping cough, the fall in the death rate preceded the use of antibiotics, and public health measures such as immunisation or improved /

improved social conditions were also of great importance.

Nowadays, despite a great abundance of antibacterial agents, bacterial infection remains very common (Sabin, 1970). Many infections become chronic and their protracted course testifies to the failure of modern therapy in such circumstances (Cluff et al, 1968). Since the antibiotics administered are often highly active against the infecting pathogen in vitro, there may be some inherent limitation of our present day regimes. For example, in chronic, localised, infection, it is clearly important that the drug is distributed to the site of infection and one finding in this thesis is that this principle applies as much at the sub-cellular level as it does in individual tissues. If the chosen antibiotic is to complement the phagocytic cells, which have rapidly ingested the infecting organisms, it must be able to penetrate and exert its antibacterial effects in the phagocytic vacuole.

Most investigation of refractory infections has centred around the degree of sensitivity or resistance exhibited by the causative organisms. In addition there have been some studies of antibiotic /

antibiotic localisation in special sites, eg. in sputum (Campbell 1970, May 1972), or in infected wounds (Ellis et al, 1975). Very few studies have focussed on the problem of the combined antibacterial action produced by the host defenses when they are "augmented" by antibiotic therapy. Perhaps the clinical immunologist, the clinical microbiologist and the clinical pharmacologist have moved too far apart. In my clinical work I have seen infected patients whose pathogen was extremely sensitive to the antibiotic administered. Furthermore it was possible to demonstrate that the drug was absorbed and that it reached the site of infection. Nevertheless some such patients did not respond to therapy. Immunological investigations before, during and after courses of antibiotics seemed to be a possible approach to the problem.

WHY STUDY PHAGOCYTIC CELLS?

The range of leucocyte activities which occur in response to invading bacteria is so great that to select for study one specific function of one group of cells may lead to false conclusions. This is /

is because the different branches of the immune system are co-ordinated in such a way that a healthy balance is maintained. For example, R. McG ., a boy of 7 with chronic granulomatous disease (page117) had raised immunoglobulins (IgG = 2600mg/100ml., IgA = 1000 mg/100 ml., IgM = 365 mg/100 ml.) and these would, to some extent, have helped to control bacterial infections. Neutropenic patients, and those with long-standing defects of intracellular killing, often show a compensatory increase of immunoglobulins. There is, however, no evidence that the reverse occurs, i.e. that phagocytic cells can increase their rate of phagocytosis or of intracellular killing in response to a primary defect of lymphocyte function.

When this project was planned it was thought that measurements of phagocytic cell function during antibiotic therapy would show increases in the antibacterial activity and that this would be attributable to the effect of antibiotics. The influx of antibiotics and of phagocytic cells from the blood stream to the site of infection had obvious similarities, and could be qualitatively compared (Chapters 2 and 3). In addition, a study of /

of the anti-bacterial activities of phagocytic cells was defended on physiological grounds, since these cells are the first to participate in the reaction to infection, comprising over 95% of the cells present in the first 24 hours of inflammation (Chapter 2, Hurley 1972).

WHAT HAPPENS TO NEUTROPHILS DURING INFECTION?

Localised infection caused by pyogenic bacteria initiates an inflammatory response which is very similar to the skin window reaction. The measurements of Chapter 2 show that normal subjects can achieve concentrations ranging from 2×10^4 phagocytic cells/ml serum (in the first 3 hours) to 4×10^7/ml serum (averaged over 24 hours). My findings indicate a slightly lower leucocyte mobilisation rate per unit area than was reported by Perillie & Finch (1964) or Senn et al, (1969). However, these groups both used autologous serum in the skin chambers, while I used plain medium 199. The serum could well have contained chemotactic factors which would increase the rate of cellular response. In contrast to the normal subjects, neutropenic patients or those with leukaemias achieve much lower cell concentrations.

Chapter 4 reports the anti-staphylococcal, phagocytic and bactericidal activity, measured using neutrophils in a concentration of 5×10^6/ml., (a concentration attainable in acute inflammatory exudates).

Both phagocytosis and intracellular killing are functions which are biologically important. I have found, as did Solberg & Hellum (1972), that during infection the intracellular killing of staphylococci is decreased without there being any effect upon phagocytosis. This phenomenon must be due to a serum factor, since it is measured in peripheral blood neutrophils which have not yet encountered bacteria in the infected site. McCall et al, (1973) studied the metabolic perturbations occurring in toxic neutrophils from patients with febrile illnesses and showed an increase of the hexose monophosphate shunt activity and of the oxygen utilisation following phagocytosis. Unfortunately their study did not include measurements of intracellular killing. They could not show any factor in the serum of these patients which produced similar metabolic changes in the neutrophils from control subjects. Whatever the cause, the reduced intra/

intracellular killing of staphylococcus might be expected to lead to a higher risk of superinfection with that organism

DO ANTIBIOTICS ENTER AREAS OF INFLAMMATION?

Chapter 3 shows that it is possible to demonstrate antibacterial activity in those inflammatory exudates which are provoked shortly after the administration of an antibiotic. It is unwise to place too much stress on the individual antibiotic concentrations in the exudates or on the differences between the various drugs examined. However, antibiotics enter the exudate before many of the phagocytic cells have accumulated. The clinical consequence would be that whenever a patient, who is receiving an antibiotic, develops some secondary focus of infection and hence inflammation, he may achieve bactericidal concentrations of the antibiotic in that site before the phagocytic cells have localised.

WHERE DO ANTIBIOTICS ACT IN VIVO?

Measurements of the anti-staphylococcal action of the five drugs studied in Chapter 5 showed that in general this process was much slower than the rate /

rate of phagocytosis. Thus unless these antibiotics can enter phagocytic cells their action would be confined to the inhibition or killing of the few bacteria which remain in the extracellular situation. Mandell (1973) believes that only those antibiotics which are highly lipid soluble (eg. rifampicin) can enter phagocytic cells, basing this view on the failure to reduce intracellular bacteria even in the presence of high concentrations of water soluble, anti-staphylococcal drugs, (intrinsic killing by the neutrophils was blocked by high concentrations of hydrocortisone). Even if a significant concentration of antibiotic penetrates the cell, its action will depend on the local intracellular conditions such as pH. The tests in the in vitro system at pH 7.4 (tables 5:1 & 5:2) may not therefore be relevant.

There is some evidence that bacteria exposed to antibiotics prior to phagocytosis may be damaged (but not killed) so that subsequent intracellular killing by neutrophils is increased. (Alexander & Good 1968, Seneca & Peer 1966). This aspect has not been followed up in this thesis since it is difficult to set up in vitro models which reflect the changing in vivo situations.

DO ANTIBIOTICS ACT ADVERSELY UPON NEUTROPHIL FUNCTION?

The initial comparisons of infected patients, both treated and untreated, suggested that, during therapy, intra-cellular killing is reduced. This occurred with various antibiotics. Furthermore, sequential tests in individual patients tended to confirm this phenomenon. Only one patient had increased intra-cellular killing while receiving an antibiotic and she had multiple pathology (Table 5:6).

It has not been possible to investigate this aspect systematically but a series of detailed studies with ampicillin have shown that oral administration of that drug to healthy subjects leads to reversible intracellular effects. The likely sequence is that some ampicillin enters the cells and causes inhibition of both myeloperoxidase and the enzymes of the hexose monophosphate shunt. Concomitantly there may be increased catalase activity. During these enzyme changes, and possibly because of them, there is a reduction of intracellular killing of staphylococci (Tables 5:7, 5:8 & 5:9).

Such /

Such intracellular effects would only have clinical significance if the killing defect affected organisms which are not sensitive to ampicillin or if the changes were irreversible after prolonged therapy. If the pathogens were ampicillin sensitive, any reduced intracellular killing by the neutrophils would be compensated for by the action of that antibiotic. Apart from the immunological effect, all these findings tend to weaken Mandell's suggestion that ampicillin and similar antibiotics cannot penetrate neutrophils. (Mandell 1973).

IS THE EFFECT OF AMPICILLIN ON NEUTROPHILS UNIQUE?

Hoeprick & Martin (1969) studied the *in vitro* effect of tetracycline, polymyxin B and rifampicin on phagocytosis (assessed morphologically) and intracellular killing (studied in a much simplified technique compared with my own). They stated that neither phagocytosis nor killing was affected by these drugs in therapeutic concentrations or even at concentration ten to one hundred times higher. As stated in Chapter 4, morphological studies do not assess phagocytosis accurately. I have analysed their data for intracellular killing using my own techniques /

techniques and find that the bactericidal indices without antibiotics ranged from 14 to 17. With the antibiotics at therapeutic concentrations the indices were 11.5, 11.6 and 8.3 for tetracycline, polymyxin B and rifampicin respectively suggesting some fall in bactericidal ability.

Forsgren et al (1974) studied the phagocytosis of baker's yeast by human leucocytes incubated *in vitro* with various concentrations of either doxycycline or tetracycline hydrochloride and showed that at concentrations exceeding 3.1 mg/l there was a significant fall in the percentage of granulocytes which were phagocytic. In addition they showed that this phenomenon occurred when healthy volunteers took tetracycline. Seneca & Peer (1966) studied the *in vitro* effect of a wide range of antibiotics upon the phagocytosis (but not intracellular killing) of *Proteus vulgaris* and *Streptococcus haemolyticus* (Group A). Tetracycline and streptomycin reduced the phagocytosis of both organisms, but only high concentrations (62.5 mg/l and above) were tested. At levels of 250 mg/l, penicillin slightly reduced phagocytosis of the streptococci.

Alexander & Good (1968) studied the effect of several /

several antibiotics *in vitro* upon the intracellular killing of *Staphylococcus aureus*. The antibiotics studied (which included ampicillin, tetracycline and erythromycin) had no effect on intracellular organisms but with penicillin G there was evidence that some organisms were damaged by the antibiotic, but not killed, and that they subsequently were more susceptible to phagocytosis. A criticism of their technique is that viable counts of intracellular bacteria alone were not made, only the combined counts which included extracellular and cell surface adherent bacteria.

DO THESE FINDINGS HAVE ANY CLINICAL SIGNIFICANCE?

Fungal infections, especially with candida species, are common complications of antibiotic therapy, particularly with penicillins (Seelig 1966). Candida infection seems to occur far more often than other similarly ubiquitous micro-organisms which are also antibiotic resistant. Human resistance to candida principally involves phagocytosis and intracellular killing via the myeloperoxidase system (Lehrer 1972) and therefore all the changes shown in Tables 5:8 and 5:9 would be expected to increase the susceptibility to /

to that fungus. The most definitive evidence in this situation would be if phagocytic defects could be shown in those individuals who develop candida superinfection following the administration of a narrow spectrum penicillin.

The common explanation for superinfection during antibiotic therapy is that drug-resistant species have been selected. This is easily demonstrable but selection may not be the only factor. Drug-resistant species would proliferate much more rapidly if there was a concomitant immunological defect. Thus Speller et al (1976) have studied the epidemiology of infection due to a gentamicin-resistant staphylococcus, in surgical and intensive care wards. Colonisation was much more likely to occur in patients receiving antibiotics. Similarly Tillotson & Finland (1968) and Philp & Spencer (1974), showed that secondary pulmonary infections following antibiotic therapy were often caused by Staphylococcus aureus, Klebseilla pneumoniae or Pseudomonas aeruginosa. Defence against these three species is in large part mediated via phagocytosis and intracellular killing.

Immunological effects of antibiotics would also cause infection to occur in sites far distant from the primary focus. Mangi et al (1973) described five patients in whom pyogenic meningitis developed while they received cephalothin for infection elsewhere. In all five cases the organism isolated from the cerebrospinal fluid (C.S.F.) was sensitive to cephalothin. Discussions of this phenomena were centred around the possible stimulant effect of sub-inhibitory concentrations of the antibiotic in the C.S.F. However, it has been stated that "something seems to be missing in the argument about the causation" (British Medical Journal, 1973). Perhaps cephalothin also affects phagocytic cell function !!

ARE OTHER IMMUNOLOGICAL RESPONSES AFFECTED BY ANTIBIOTICS?

There are scattered references in the literature to other immunotoxic effects of antibiotics (Tarnowski & Batko 1973). These have mainly concerned reduced antibody formation (in experimental animals), following administration of several antibiotics, including penicillin, tetracycline and chloramphenicol (eg. Slanetz 1953, Watson, 1959, Siefert et al /

Siefert et al, 1971). Stevens (1953) showed that penicillin and several tetracyclines given to rabbits slowed the rate of removal of radio-labelled bovine gamma globulin. In addition, it has been known for many years that antibiotic therapy of acute infections will reduce the antibody levels ultimately attained (Daikos & Weinstein, 1951, Siegel et al, 1961). All these reports raise the suspicion that antibiotics interfere with the human response to infections. Recognition of the clinical sequelae of such "immunotoxic" effects might well be disguised as antibiotic resistant or chronic granulomatous infections. A further difficulty in interpreting such phenomena is that many in vitro tests of immune function involve short term tissue cultures in which penicillin and streptomycin are routinely added to the test system as prophylaxis against contamination.

CONCLUSIONS

A series of experiments have been carried out to compare the efficiency of human phagocytic cells and of several antibiotics in controlling staphylococcal infection. Neutrophils, 'skin window macrophages', and several antibiotics can be demonstrated in acute inflammatory exudates but the kinetics of their localisation in the test site is very different. In vitro models have been exploited to measure the rate of phagocytosis and of intracellular killing so that these functions of phagocytic cells can be compared with the inhibitory or bactericidal actions of antibiotics. In general phagocytosis is very rapid, much more so than the action of antibiotics. Intracellular killing is a slower process roughly comparable to the bactericidal effects of anti-staphylococcal agents such as cloxacillin.

An unexpected finding was that when administered to human subjects in vivo certain antibiotics appeared to have adverse effects upon neutrophil function. In the case of ampicillin this was studied by serial measurements of intracellular killing, neutrophil /

neutrophil myeloperoxidase and catalase in normal subjects who received a single dose of 500 mg. This showed that ampicillin has actions upon the enzymes which would adequately explain a diminution of intracellular killing.

There are several clinical situations which would be explicable by such *in vitro* phenomena, and some defects of the conventional explanations have been summarised. If other antibiotics have 'immunotoxic' effects similar to ampicillin a change in present day antibiotic policies would be indicated. At the very least the findings provide further important reasons for reducing the over prescribing of antibiotics and for deprecating their use in mild, self-limiting infections.

APPENDIX 1

COMPARISON OF VARIOUS CYTOCHEMICAL AND FUNCTIONAL PROPERTIES OF MONOCYTIC CELLS IN SKIN WINDOWS AND IN THE PERIPHERAL BLOOD

These experiments were performed to identify the origin of "skin window macrophages". Since in the mouse model, macrophages are derived from blood monocytes (van Furth and Cohn, 1968), we studied human blood monocytes (see method 2, page 103) as well as skin window cells during *in vitro* culture. Table A:1 shows the results and confirms the great similarity between the two types of cell. Taken in conjunction with *in vivo* labelling studies (Whitelaw 1975) and with the animal models, it seems almost certain that skin window macrophages in man are derived from blood monocytes.

Table A:1

CHARACTERISTICS OF HUMAN BLOOD MONOCYTES AND SKIN MACROPHAGES

	Peripheral blood monocytes		Skin macrophages	
	Incubation time		Incubation time	
	6 hr	48 hr	6 hr	48 hr
	%	%	%	%
Phagocytosis [x]	91.3	87.0	97.0	82.8
Pinocytosis [x]	68.0	95.0	69.4	88.1
Esterase [x]	97.0	94.0	99.5	98.0
Peroxidase [x]	88.0	73.9	80.0	63.0
Thymidine-^{3}H [x] labelling	0	0	0	0

[x] See appendix 6 , page 215.

Appendix 2

QUALITY CONTROL OF GENTAMICIN ASSAYS*

True value mg/l	Our value mg/l	% error
15.7	16.0	+ 2
5.4	3.9	-26
2.1	1.1	-48
8.4	6.0	-29
2.1	1.1	-48
8.4**	5.5**	-36

These results show that my assay system is fairly accurate at the upper limit of the therapeutic range. However, lower concentrations are underestimated and there is a degree of 'drift'.

* See Reeves & Bywater (1975)

** Serum also contained lincomycin.

APPENDIX 3

ANTIBIOTICS IN INFLAMMATORY EXUDATES - CORRECTION FACTORS

The weight of exudate produced in each hourly collection after antibiotic administration varied from about 5 mg to 20 mg. Initially these were assayed against standards placed on identical assay discs which were then fully saturated (weight taken up was around 35 mg). Subsequently a series of assays were performed using standards of 10 mg, 20 mg, 25 mg or 30 mg and when these were plotted an approximate correction factor could be obtained. Figure A, 3:1 shows a typical correction graph (for cephalexin). It is clear that a level read off using 30 mg standards from an exudate sample weighing 10 mg, would be grossly underestimated. Figure A 3:2 shows these results for cephalexin by plotting the zone of inhibition against weight of standard at each level of antibiotic. Clearly these are not straight line relationships and the use of simple multiplication to correct for the differing exudate weights would be quite wrong.

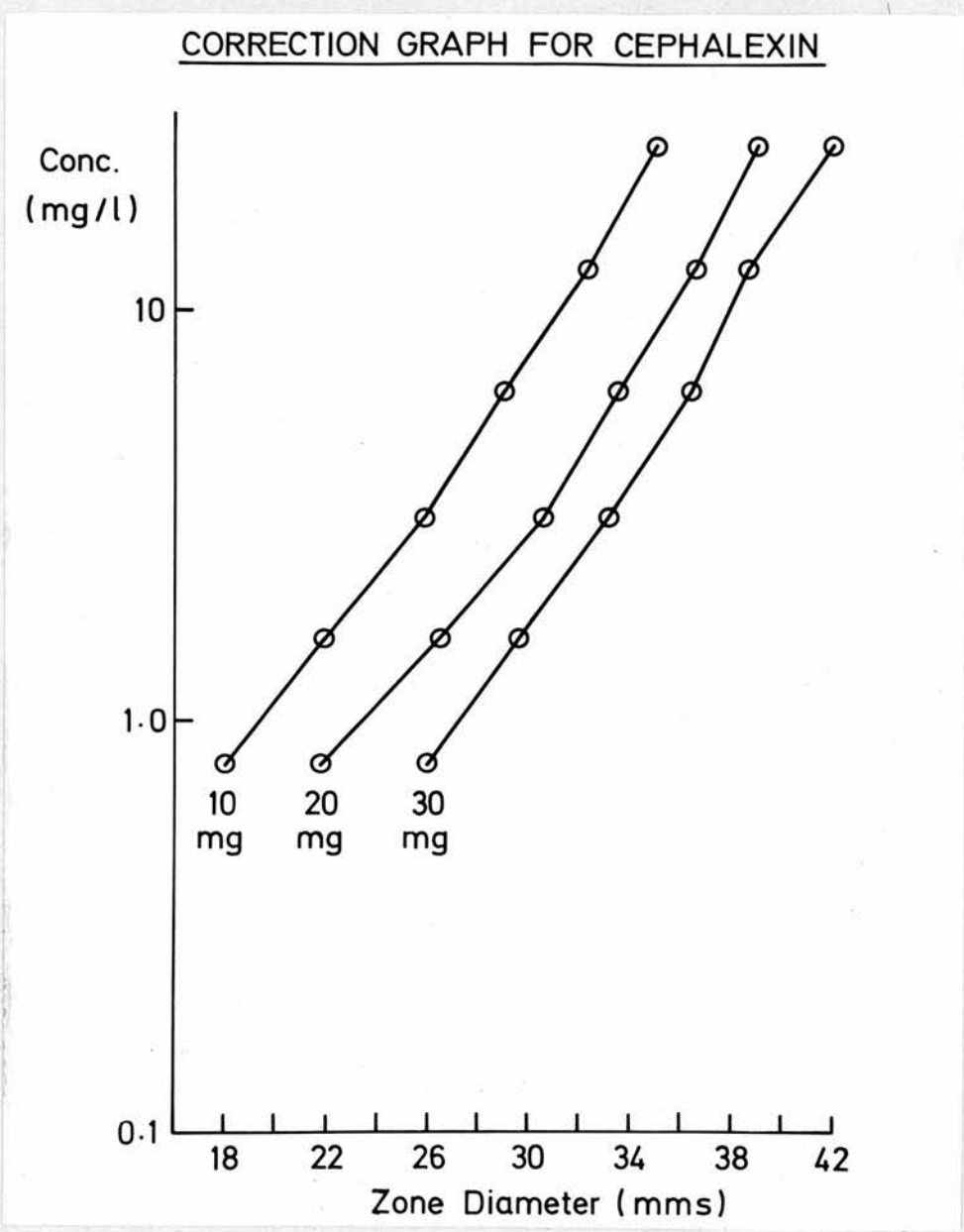


Figure A 3:1 Log-plot of cephalexin concentration against zone diameter. The original result for an unknown sample is read off and the zone diameter (D) for that concentration (with a 30 mg standard) is found. The nearest weight of standard to the unknown is then checked for a diameter of D and the corrected value is read off.

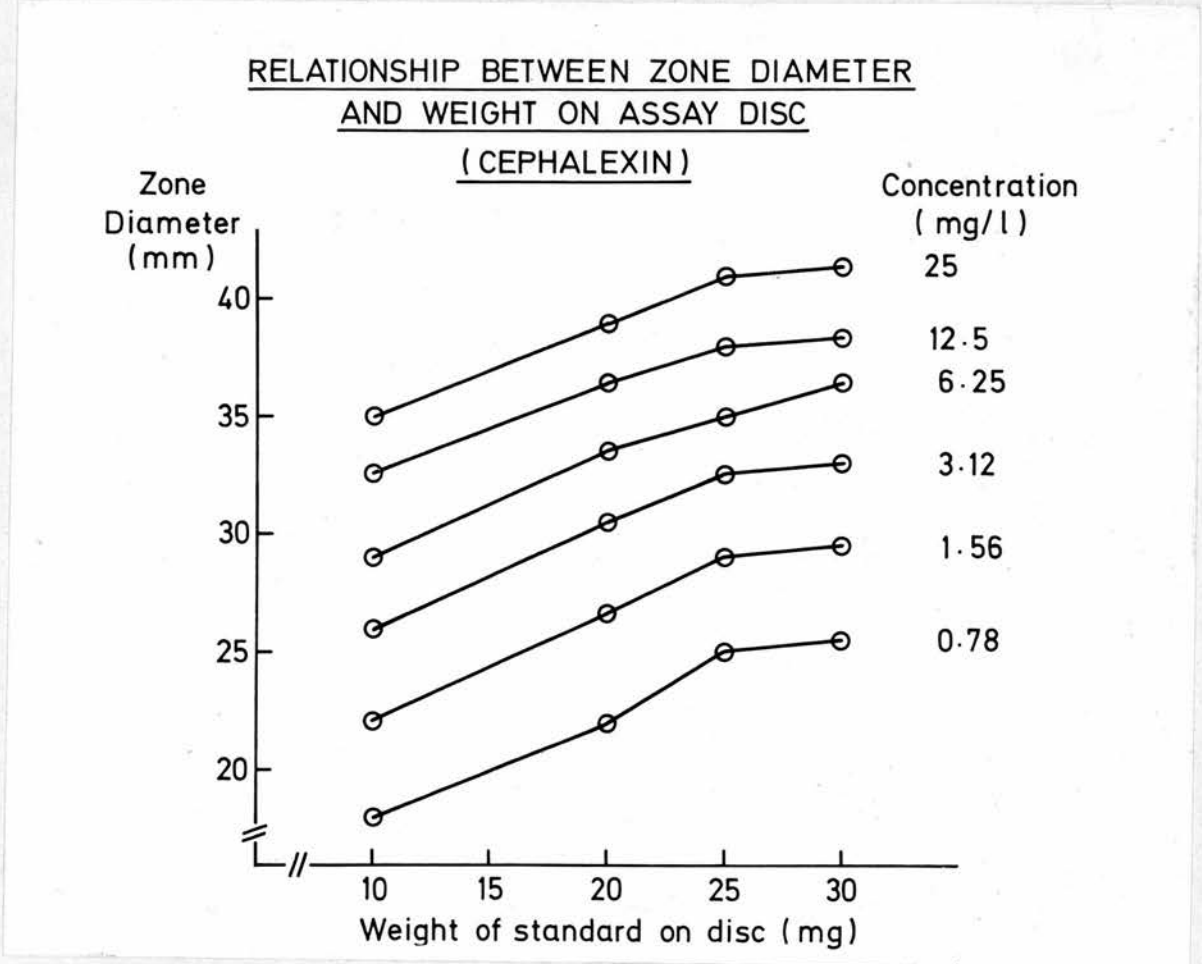


Figure A 3:2 Graphs of zone size against weight of standard for various concentrations of standard. Note the overall plateau effect on zone size after weights of 25 mg.

APPENDIX 4 (a)

SOURCES OF MEDIA, REAGENTS ETC.

Nutrient agar	Oxoid Ltd.
Buffered physiological saline	" "
Antibiotic medium no. Z	" "
Hank's solution xl(+glutamine)	Gibco Biocult Ltd.
Medium 199	Microbiological Associates Baltimore.
Gelatine	Difco Laboratories.
Ficoll	Pharmacia, Uppsala, Sweden
Dextran 500	" " "
Heparin (without preservative)	Evans Medical Ltd. London.
Basic Fuchsin	B.D.H. Ltd. Poole.
Sodium Cacodylate	" " "
Nitro blue tetrazolium	Sigma London Chemical
Alpha naphthyl butyrate	Ltd.
AB serum - pooled from 2 donors	Blood Transfusion Service, Edinburgh.
Falcon tubes	Falcon Plastics Ltd. via Dynatech, Billingshurst.
Microplates (M34R)	Cooke Instruments via Dynatech
Filter paper discs (12mm)	Mast Laboratories Ltd.
Antibiotic Standards:	
a) Ampicillin, Penicillin & Cloxacillin	Beecham Research Labs. Brentwood.
b) Fucidin & mecillinam	Leo Laboratories Ltd. London
c) Tobramycin, gentamicin & cephalexin	Eli Lilly & Co. Ltd.
d) Clindamycin	Upjohn Ltd. Crawley.

APPENDIX 4 (b)

APPARATUS USED IN STUDIES

Electric colony counter -	Chiltern (3L30), via Macfarlane Robson, Glasgow.
Microscope	Leitz Laborlax, Leitz, W. Germany.
CO_2 incubator	Assab Ltd., Sweden
Centrifuge	Christ Bench Centrifuge with 14 cm swingout head.

Preparation of Kaplow's reagent (see page 21)

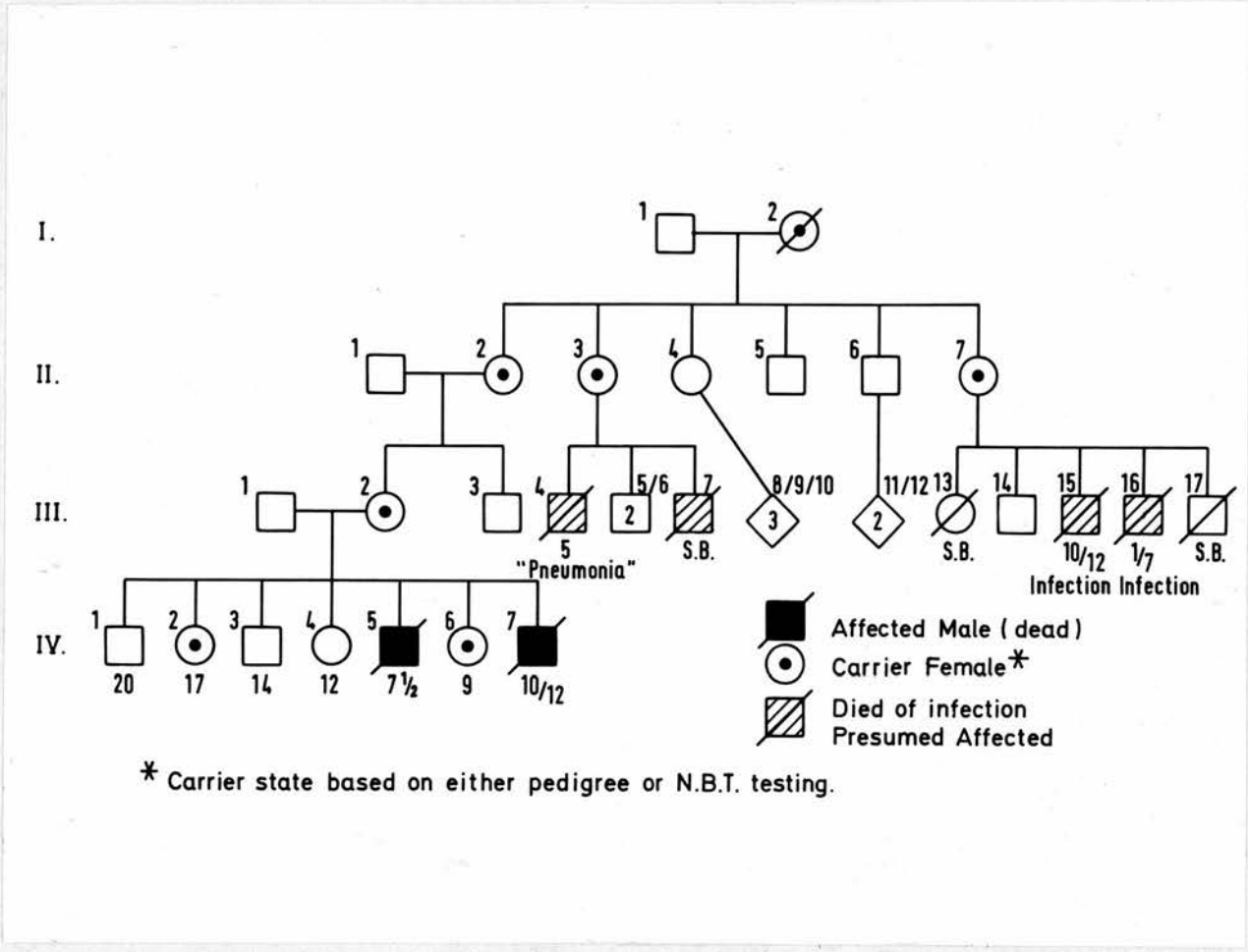
1.	30% ethyl alcohol	100 ml.
2.	Benzidine dihydrochloride (Fluka, Buchs)	0.3 grams
3.	0.132 molar (3.8% W/V) Zn $SO_4 \cdot_7 H_2O$	1.0 ml.
4.	Sodium acetate (Na C_2 H_3 $O_2 \cdot_3 H_2O$) (Merck)	1.0 gram.
5.	3% hydrogen peroxide	0.7 ml.
6.	1.0 normal, NaOH	1.5 ml.
7.	Safranin (Merck)	0.2 grams

Instructions.

Add reagents in the above order from 1 onwards mixing well each time. There may be inert residue in the benzidine which does not dissolve. Final PH should be 6.00 ± 0.5.

Filter solution through ordinary filter paper and store at room temperature keeping away from excess light. Can be used for up to six months.

Appendix 5



Pedigree of the family with chronic granulomatous disease described in Chapter 4 (page 117). R. McG., was IV, 5, his sister was IV, 1 and his mother was III, 2. (see table 4:8).

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Mrs. Marjory Grant and Dr. R.A. Harkness carried out the studies of leucocyte myeloperoxidase activity and gave me permission to report these in chapter 5. Mr. W. Lutz and Miss Celina Marques studied the initial results and suggested suitable statistical techniques for comparisons of phagocytic and bactericidal activities between different tests.

Professors /

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APPENDIX 6

i) Details of chambers used for collecting leucocytes from skin windows.

Chamber a was made from the metal top of a glass universal by embedding two ½ inch syringe needles (23 G diameter) so that one needle opened at the top, while the other was at the open end and close to the skin surface when in position. The needles were fixed in position by an epoxy-resin glue (araldite). Chamber b was supplied by Dr. W.I.H. Sheldon of the Johns Hopkins University and had the advantage of a wider flange at the skin surface which minimised leakage of fluid. The internal diameter of this chamber was 7mm and fluid was exchanged via polypropylene tubing attached to metal needles. The internal volume of chamber a was 2.1 ml and of chamber b was 0.5 ml.

ii) Maintenance of micro-organisms.

The organisms used for antibiotic assays and the Staphylococcus type 42b were maintained on nutrient agar slopes in glass bijou bottles. These organisms were sub-cultured every fortnight, incubated for 18 hours at 37°C and then stored at 4°C. On the day prior /

prior to use they were inoculated into nutrient broth using a standard bacteriological loop before overnight incubation. For antibiotic assays between 0.05 ml and 0.5 ml of the overnight cultures (dependent on the degree of growth, assessed visually) was added to each 100 ml of molten agar (pages 56 and 57).

For studies of phagocytosis and intracellular killing the overnight cultures of Staphylococcus type 42b were centrifuged (3000 rpm for 10 minutes), washed twice in sterile saline and resuspended in Hank's solution plus 0.1% gelatine. A 1 in 10 dilution of this solution contained approximately 1×10^7 staphylococci per ml. (page 103)

SOURCE OF MICRO-ORGANISMS

ORGANISM	SOURCE
Sarcina lutea	N.C.T.C. no 8340
Corynebacterium xerosis	Leo Laboratories Ltd.
Serratia marcescens	Dr. J.D. Sleigh, Glasgow
Staphylococcus type 42b	Professor R. van Furth, Leiden
Staphylococcus albus	" " "

iii) Antibiotic assay in mixed leucocytes

Mixed /

Mixed leucocytes were prepared by dextran sedimentation as described on page 103 and brought to a concentration of 2 x 10^7 cells/ml in medium 199. 150 µl volumes were added to the wells of round-bottomed microtitre plates (M34R) and at various times 50 µl of medium containing either ampicillin or tobramycin was added to give a final concentration in the cell mixture of 2.5 mg/l. After incubation for varying periods up to 4 hours the samples were centrifuged at 500 x g for 15 minutes and the supernatants were separated from the cell pellets. Both supernatants and cell pellets were assayed and the cell concentration was expressed as a percentage of the supernatant value (table 5:3, page 145).

iv) Method for estimation of leucocyte myeloperoxidase and catalase

(Kindly performed by Mrs. Marjory Grant & Dr. R.A. Harkness)

Mixed leucocytes were obtained from 8 ml heparinised blood (10 units heparin per ml) by dextran sedimentation as described on page 103. They were then washed twice with buffered saline and centrifuged at 100 x g for ten minutes. Contaminating erythrocytes were removed by differential lysis using /

using 2 ml of 0.2% sodium chloride and after 15 seconds, hypertonic saline was added to restore isotonicity. If necessary, this process was repeated. The cell mixture was spun at 100 x g for 5 minutes and the cell pellet was resuspended in 0.5 mls buffered saline. The leucocytes were then lysed by freezing (to - 20°C) and thawing three times. Debris was removed by centrifugation at 800 x g for 20 minutes at 4°C.

Myeloperoxidase (M.P.O.) activity was assayed at pH 6.0 using orthodianisidine (B.D.H.) as substrate in the presence of an excess of hydrogen peroxide. The activity was based on the oxidation of the substrate, measured spectrophotometrically at 460 nm, and was expressed as M.P.O. units per ug protein.

Catalase activity was assayed by measurement of the rate of enzyme-catalysed destruction of hydrogen peroxide (1.25×10^{-2} M) in 0.01 M phosphate buffer, using ultra violet spectrophometry. The activity was expressed as moles catalase x 10^{-13}/ug protein.

Protein concentrations used in the standardisation of both enzyme activities were determined by the method of Lowry et al, 1952.

v) Method for performing the nitro-blue tetrazolium test

(Kindly performed by Dr. K.C. Watson)

One /

One tenth ml heparinised whole blood was mixed in microplate wells with an equal volume of 0.2% nitro-blue tetrazolium (N.B.T.) (Sigma) in saline. Further wells contained similar volumes of the blood/NBT mixture but in addition 2 loopfuls of an overnight culture of Staphylococcus aureus were added. After mixing, the preparations were incubated at 37°C for 20 minutes and films were made on microscope slides before air drying. They were fixed in formo-ethanol for one minute and then counter-stained for 10 minutes using a 1 in 20 aqueous dilution of Giemsa (Merck).

The stained films were examined under high power magnification and the number of N.B.T. positive cells (containing dark blue formazan deposits in the cytoplasm) out of 100 neutrophils counted was recorded.

vi) Cytochemical methods used to obtain data of Table A:1 (page 184)

Separated monocytes (see pages 103 & 104) were suspended in medium 199 plus 20% inactivated newborn calf serum (N.B.C.S.) (Grand Island Biological Co., Grand Island, New York). The final cell concentration was 2.0×10^6 per ml and 1 ml aliquots were added to Leighton tubes containing 10 x 35mm coverslips.

Each /

Each tube was gassed with 5% CO_2 in air, tightly stoppered and then incubated at 37°C for 6 or 48 hours. The 48 hour preparations had a medium change at 24 hours.

Skin macrophages were collected on coverslips (page 26, method 1) and were cultured in 50mm Petri dishes containing 2 ml of medium 199 (+ 20% N.B.C.S.) in a 5% (O_2 incubator.

Pinocytosis:

To study this activity in vitro the preparations were cultured throughout in medium 199 + 20% N.B.C.S. which also contained 100 ug/ml dextran sulphate (M.W. 500.000, Pharmacia Uppsala, Sweden). After air drying and fixing in the usual way the preparations were stained for 4 minutes with toluidine blue (1% in 30% methanol). The proportion of positive cells (containing many blue stained pinocytic vesicles) was assessed under high power.

Esterase activity:

The air dried preparations were fixed in formalin vapour for 60 seconds, washed in distilled water and dried. The horizontal slides were then covered with esterase reagent for 25 minutes. The reagent was prepared by mixing 0.8 ml Basic Fuchsin solution (1.0 /

(1.0 G in 30 ml 2N Hydrochlorine acid) with 0.8 ml sodium nitrite solution (1.0 G $NaNo_2$ in 30 ml water) After 30 seconds 4.0 ml of 0.40 molar sodium cacodylate solution was then added and the pH was checked. More of the sodium cacodylate solution was then added until the pH was 6.0 and the volume was brought to 15 ml with distilled water. Then 0.15 ml of 10% tween 20 was added. This solution was added to 2 ml of 0.52% (w/v) alpha naphthyl butyrate (Sigma) in dioxane mixed with 3 ml of methanol. Distilled water was added to a final volume of 40 ml. The reagent was used for up to 6 hours. After 25 minutes in the reagent at room temperature the preparations were rinsed, dried and counter-stained with dilute Giemsa. The proportion of cells with brown stained cytoplasm was assessed under high power.

Peroxidase activity:

The air dried preparations were fixed in 10% formalin in absolute alcohol for 1 minute, rinsed in tapwater and dried. Kaplow's reagent containing 0.3% (w/v) benzidine dihydrochloride was poured over the horizontal slides and washed off after 30 seconds. This reagent is stable for up to 1 month. After /

After drying, the slides were counterstained with safranin, washed gently and dried. The peroxidase positive cells contained many black cytoplasmic granules.

In vitro labelling with 3^H thymidine:

For these studies methyl-labelled tritiated thymidine (New England Nuclear Corp., Boston, Mass. specific activity 6, 7 c/m.mole) was in the medium for the whole duration of culture in a final concentration of 0.1 Curie/ml. In addition all the solutions used to prepare the cell suspensions from blood contained the labelled thymidine. After careful washing and fixation in absolute methanol the slides were dried and prepared for autoradiography as previously described (van Furth et al, 1973), using K5 nuclear emulsion (Ilford Ltd., Essex England) and Kodak D19 developer (Kodak Ltd., England). All cells with more than 5 grams overlying the nucleus were reported as positive.

vii) Methods for studies of antibiotics and phagocytic cells in vitro. (page 147, 1.)

These experiments were performed using mixed leucocyte preparations obtained by method 1 (page 103). Phagocytosis of Staphylococcus 42b was performed using /

using the established technique (method 2 page 106) and a 30 minute period of incubation. The mixtures were then centrifuged at 100 x g for 5 minutes and the cell button was washed twice with heparinised physiological saline before resuspension in Hank's solution plus 10% AB serum to a cell count of 5 x 10^6/ml. The mixture was then split into two and ampicillin in distilled water (750 mg/l) was added using a Hamilton micro syringe to give a final concentration in one of the cell mixtures of 5 mg/l. The two preparations were incubated for a further 2 hours and were sub-sampled for viable counting as described on page 110. The bactericidal index was calculated in the usual way. In one experiment the preparation containing ampicillin was set up in duplicate and each sub-sample from one tube was treated with penicillinase (E.C. 3.5. 2.6) of staphylococcal origin, at a concentration of 100 units/ml. In addition the effect of ampicillin alone was studied in a cell free system.